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Foot-and-mouth disease virus (FMDV) causes an acute disease that can be lethal for adult laboratory mice

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Abstract

Foot-and-mouth disease virus (FMDV) is a picornavirus that causes an acute vesicular disease of cloven-hoofed animals. This virus continues to be threat to livestock worldwide with outbreaks causing severe economic losses. However, very little is known about FMDV pathogenesis, partially due to the inconveniences of working with cattle and swine, the main natural hosts of the virus. Here we demonstrate that C57BL/6 and BALB/C adult mice are highly susceptible to FMDV infection when the virus is administered subcutaneously or intraperitoneally. The first clinical signs are ruffled fur, apathy, humped posture, and wasting, which are followed by neurological signs such as hind-limb paralysis. Within 2–3 days of disease onset, the animals die. Virus is found in all major organs, indicating a systemic infection. Mice developed microvesicles near the basal layer of the epithelium, event that precedes the vesiculation characteristics of FMD. In addition, a lymphoid depletion in spleen and thymus and severe lymphopenia is observed in the infected mice. When these mice were immunized with conventional inactivated FMDV vaccine, they were protected (100% of vaccinated animals) against challenge with a lethal dose of FMDV. The data indicate that this mouse model may facilitate the study of FMDV pathogenesis, and the development of new effective vaccines for FMD.

Keywords: Foot-and-mouth disease virus; Mice; Lymphopenia

Introduction

Foot-and-mouth disease virus (FMDV) is a member of the family *Picornaviridae*, genus *Aphthovirus*, that causes a highly contagious and economically devastating disease of cloven-hoofed livestock, characterized by the appearance of vesicles on the feet and mouth (Bachrach, 1978; Rowlands, 2003; Sobrino and Domingo, 2004). The economic and social impact of foot-and-mouth disease (FMD) can be catastrophic when an outbreak occurs in FMD-free countries populated with immunologically naive animals. This is the case of the FMD outbreaks in Taiwan in 1997 and in United Kingdom in 2001 resulted in the culling of millions of infected and in-contact, susceptible animals, and billions of Euros in direct and indirect costs (Gibbens et al., 2001; Knowles et al., 2001; Yang et al., 1999).

Similar to other RNA viruses, FMDV viral populations follow a quasispecies dynamics as a result of the high mutation rates during genome replication (reviewed in (Domingo et al., 2001). This genetic heterogeneity and diversification is reflected in its serological diversity, with seven antigenically distinct serotypes, O, A, C, South African Territories (SAT) 1, SAT 2, SAT3, and Asia 1 (Pereira, 1981). Immunity to one serotype does not provide protection against the others complicating the design of vaccines. In addition, many subtypes within one serotype,

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which evoke partially cross-protective immune responses, have been identified. This level of heterogeneity is also reflected in the severity of resulting disease in natural hosts that varies with the virus strain (reviewed in (Donaldson, 2004). By comparing reactivity with monoclonal antibodies, it has been shown that FMDV populations are composed of a continuum of antigen variants, with amino acid substitutions affecting several antigen sites on the viral particle (Mateu, 1995). The design of effective vaccines is made more difficult by this antigenic heterogeneity since vaccines must match the antigenic properties of the circulating viruses.

The mechanisms underlying the pathogenesis and the determinants of protective immunity to FMDV are not well understood. A basic understanding of FMDV pathogenesis and the type of immune response needed to confer full protection are needed. Studies involving the natural hosts of FMDV are limited by the complexity of the system and the lack of reagents to define components of the immune response. Even use of guinea pigs meets with considerable limitations regarding host genetics and reagents to characterize immune responses (Balamurugan et al., 2003; Bittle et al., 1982; Dunn et al., 1998; Huang et al., 1999; Knudsen et al., 1979; Nunez et al., 2001). To circumvent some of these problems, an adequate system would be the use of adult mice because of the knowledge of its genetics and its manageability. Mice have been used as an alternative to the natural host to study many viral diseases such as those associated with infection by Ebola virus (Bray et al., 1998), Hantaviruses (Wichmann et al., 2002), Dengue virus (Huang et al., 2000), Venezuelan equine encephalitis virus (Gleiser et al., 1961; Jackson et al., 1991), and Herpes simplex virus (Hayashi et al., 1986), among others. The use of murine models for these diseases has helped elucidating several mechanisms of the pathogenesis of these viruses, which are difficult to achieve using the natural hosts.

Skinner (1951) demonstrated that unweaned mice could be readily infected intraperitoneally with most serotypes of FMDV. Since then, unweaned mice have been used extensively in many laboratories for the detection and titration of virus and for serum neutralization tests (Heatley et al., 1960; Skinner et al., 1952; Subak-Sharpe, 1961). FMDV induces a rapidly fatal infection in these mice characterized by muscular paralysis of the limbs, showing severe macroscopic degenerative changes in the limb muscles, in other skeletal muscles, and in the myocardium (Platt, 1956). However, this susceptibility wanes rapidly with increasing age, so that mice aged between 3 and 4 weeks rarely become critically ill. There appears to be in agreement with the literature that normal adult mice do not show disease symptoms even when inoculated with very large doses of FMDV (Borca et al., 1986; Lopez et al., 1990; Piatti et al., 1991; Subak-Sharpe, 1961). A subclinical infection was produced in some strains (CH3, Swiss, BALB/c and nude) of adult mice inoculated with FMDV serotype O or A (Collen et al., 1984, 1989; Fernandez et al., 1986). Viraemia lasted for 48 to 72 h post-inoculation and production of neutralizing antibodies coincided with virus clearance (Borca et al., 1986; Collen et al., 1984; Lopez et al., 1990). This murine model system has been used to study some aspects of the immune response to FMDV (Perez Filgueira et al., 1995; Piatti et al., 1991; Wigdorovitz et al., 1997). However, it is less suitable for the study of the role of the immune system and host genetic factors involved in the pathogenesis of FMDV due to the lack of symptoms associated with the infection. Here, we report that several serotypes and viral variants of FMDV are lethal for the adult inbred mouse strains, C57BL/6 and BALB/C. The virus spread in almost all organs tested, causing a systemic infection. In addition, the animals show a severe lymphopenia as well as microscopic lesions in several organs similar to those described in the natural hosts. Furthermore, mice vaccinated with a classical FMDV inactivated vaccine show complete protection against a lethal dose of FMDV.

Results

FMDV serotype C causes a lethal infection in adult laboratory mice

It has been previously described that a viraemia could regularly occur in adult mice after intraperitoneal inoculation with different strains of FMDV, although mice did not shown clinical symptoms or die (Fernandez et al., 1986). In an attempt to develop an adult mouse model for FMDV in which mice show disease symptoms, we tested the susceptibility of different laboratory mouse strains to FMDV C-S8c1. Adult C57BL/6, BALB/C, SJL/J and Swiss mice (8 weeks old) were infected in the left rear footpad (LRFP) with 10^5 PFUs of FMDV C-S8c1. Since an antiviral protective response is highly dependant on the migration of different components of the immune response to the site of inoculation, we chose footpad sc inoculation to facilitate virus spread without early intervention of the immune system that could lead to virus elimination. Under these conditions, mouse strains showed different susceptibility to FMDV (Fig. 1). SJL/J mice did not show any symptoms or death following viral infection. Swiss, BALB/C and C57BL/6 mice were susceptible to infection and showed a similar disease course at different times post-inoculation. Disease was characterized by apathy, ruffled fur, humped posture, and mild wasting at 24 h post-inoculation. At late stages of the disease, the animals developed neurological signs such as ataxia in the hindlimb. The quick progression of the disease led to death of most animals within 48 to 72 h post-inoculation (Fig. 1). LD_{50} values were 50 PFU for C57BL/6, 10^4 PFU for BALB/c and 10^6 PFU for Swiss mice; SJL/J mice did

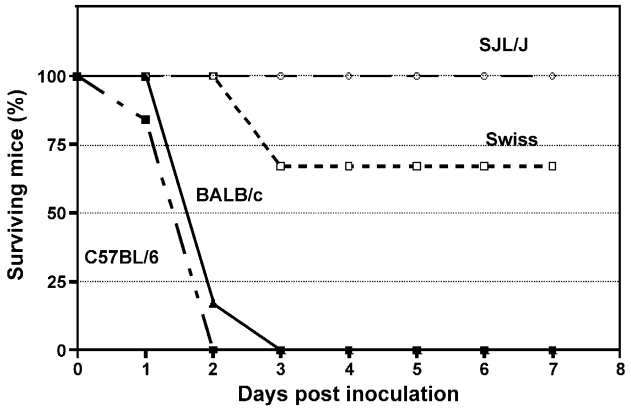

Fig. 1. Susceptibility of adult mice to FMDV infection. Different strains of laboratory mice (8 weeks old, 6 mice per group) were subcutaneously inoculated in the footpad with 10^5 PFUs of FMDV C-S8c1. The mice were observed every 4 h for the first 48 h and every 24 h for 7 days. Except for SJL/J, all other animals were susceptible to infection. C57BL/6 and BALB/c showed similar disease course. Procedures are detailed in Materials and methods.

not show any clinical symptoms associated with FMDV infection (Fig. 2). Since C57BL/6 mice showed the highest susceptibility to FMDV, this mouse strain was used for further experiments. In order to evaluate the influence of the route of inoculation in the outcome of the disease, the virulence of FMDV C-S8c1 in C57BL/6 mice was also determined after ip inoculation. The progression of the disease led to death of most animals within 48 to 72 h However, C-S8c1 was more virulent after ip inoculation, showing a LD_{50} of 10 PFU. Since the natural route of infection in the natural host is by exposition to aerosols, we carried out additional experiments in which mice were infected by the oronasal route. These mice, after infection with the highest amount of virus used (10^6 PFUs), did not show any symptom or death associated with viral infection. This indicates an influence of the inoculation route in the outcome of the disease.

Replication and spread of FMDV C-S8c1 in the mouse

To determine the dissemination of the virus beyond the footpad to serum and other tissues, time course studies were performed and the virus titer in serum and various tissues (draining lymph node (popliteal) [PLN], spleen, thymus, pancreas, liver, lung, heart, and brain) was determined at different times post-inoculation with 10^4 PFU of C-S8c1 in the LRFP (Fig. 3). Viral titers in serum peaked between 24 and 36 h post-inoculation (hpi) at 10^6 to 10^7 PFU/ml (Fig. 3A). The first tissue to be reached by the virus was the liver (Fig. 3B), where virus was detected as early as 4 hpi (10^2 PFU/g), with viral titers increasing thereafter until death (reaching titers of 10^4 PFU/g). By 8 hpi, virus was detected in spleen, pancreas, heart and thymus, reaching

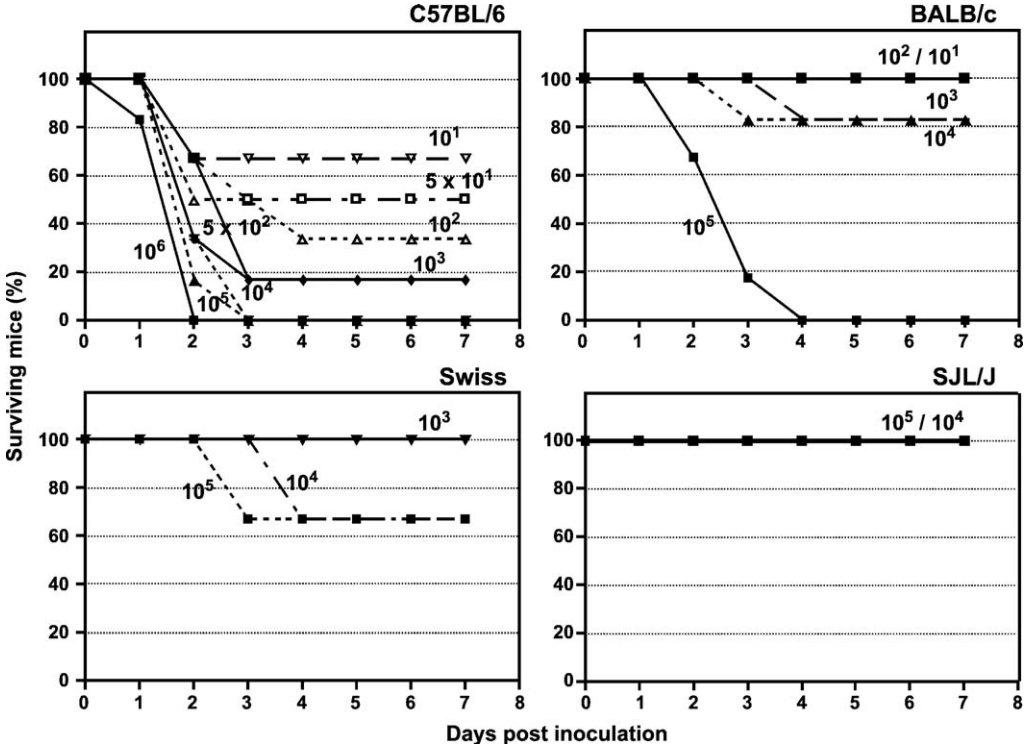

Fig. 2. Survival curves of C57BL/6, BALB/c, SJL/J, and Swiss mice infected with FMDV C-S8c1. Mice (8 weeks old, 6 mice per group) were subcutaneously injected in the footpad with 10-fold dilutions of FMDV C-S8c1; the number of PFUs inoculated is indicated on each survival curve. Values shown together indicate that the survival curves are superimposable. LD_{50} values re given in the text, and procedures are detailed in Materials and methods.

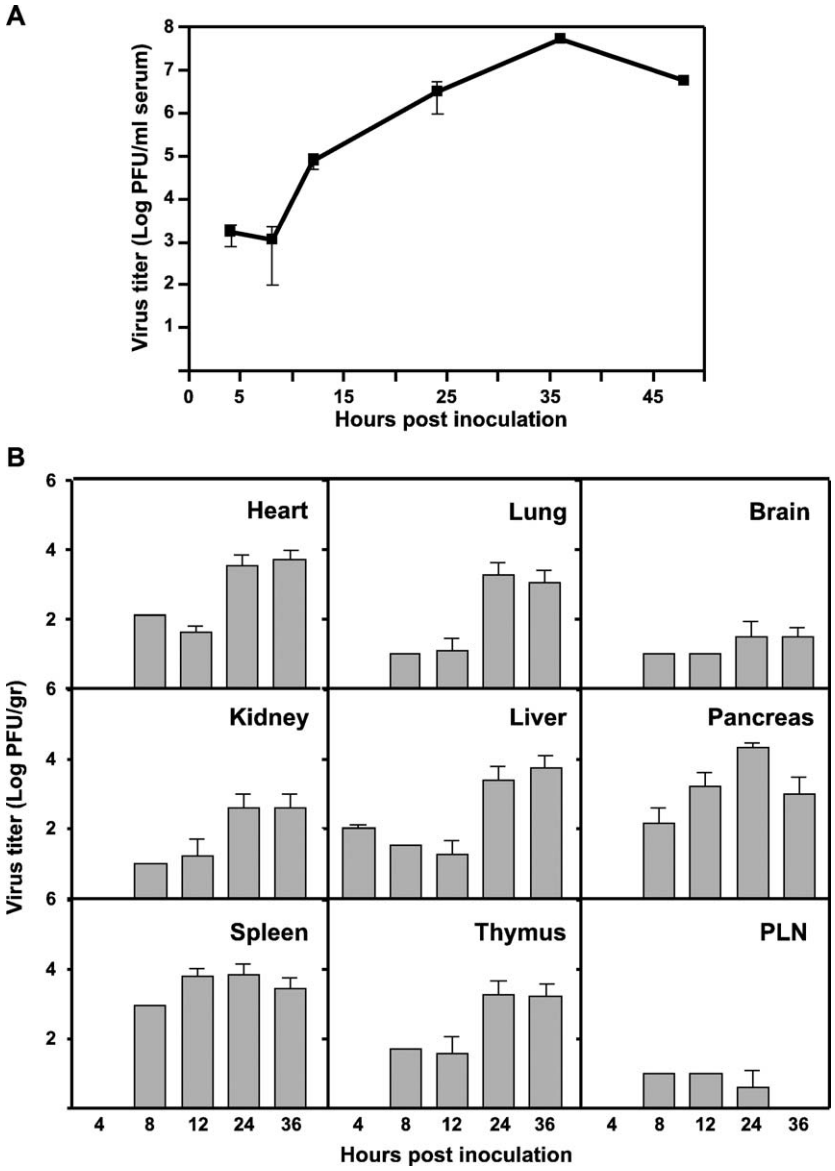

Fig. 3. FMDV C-S8c1 titers in serum and several organs of C57BL/6 mice. Mice (8 weeks old) were inoculated in the LRFP with 10^4 PFUs of FMDV C-S8c1. (A) Viral titers in serum were determined as described in Materials and methods. Each point represents the average of the viral titer of four animals. The detection limit was approximately 10 PFUs. Data are given by PFU/ml ± SD. (B) At the indicated times post-inoculation, four mice per group were sacrificed and heart, lung, brain, kidney, liver, pancreas, spleen, thymus, and draining lymph node (PLN) were harvested for viral titer determinations. The detection limit was approximately 10 PFUs. Standard deviations are given. Procedures are detailed in Materials and methods.

maximum values at 24 hpi (10^4 PFU/g), and decreasing by 36 hpi (10^3 PFU/g). Viral infectivity was below the limit of detection (10 PFU/g) in the lung and brain until 24 hpi (10^3 and 10^2 PFU/g, respectively), suggesting a slower virus spread to these tissues (Fig. 3B). Virus was detected in all the organs tested, indicating a systemic infection. In general, virus failed to clear from any of the organs analyzed, and remained at or near peak titers until the death of the animals. The only exception was the PLN in which virus replication was detected at 24 hpi (100 PFU/g) with a drop in titer below the limit of detection by 48 hpi (Fig. 3B). These results suggest that the virus does not spread to the PLN, and that replication may be taking place mainly at or near the inoculation site, and then spreading throughout the body.

Susceptibility of C57BL/6 mice to other FMDV serotypes and variants

To determine whether C57BL/6 mice were susceptible to FMDV of different serotypes, C57BL/6 mice were inoculated in the LRFP with tenfold serial dilutions of FMDV A22 and SAT-1. Mice infected with SAT-1 showed similar symptoms to those shown after infection with FMDV C-S8c1, with an LD_{50} of 10 PFU, similar to the LD_{50} for C-S8c1 (Fig. 4A). However, mice inoculated

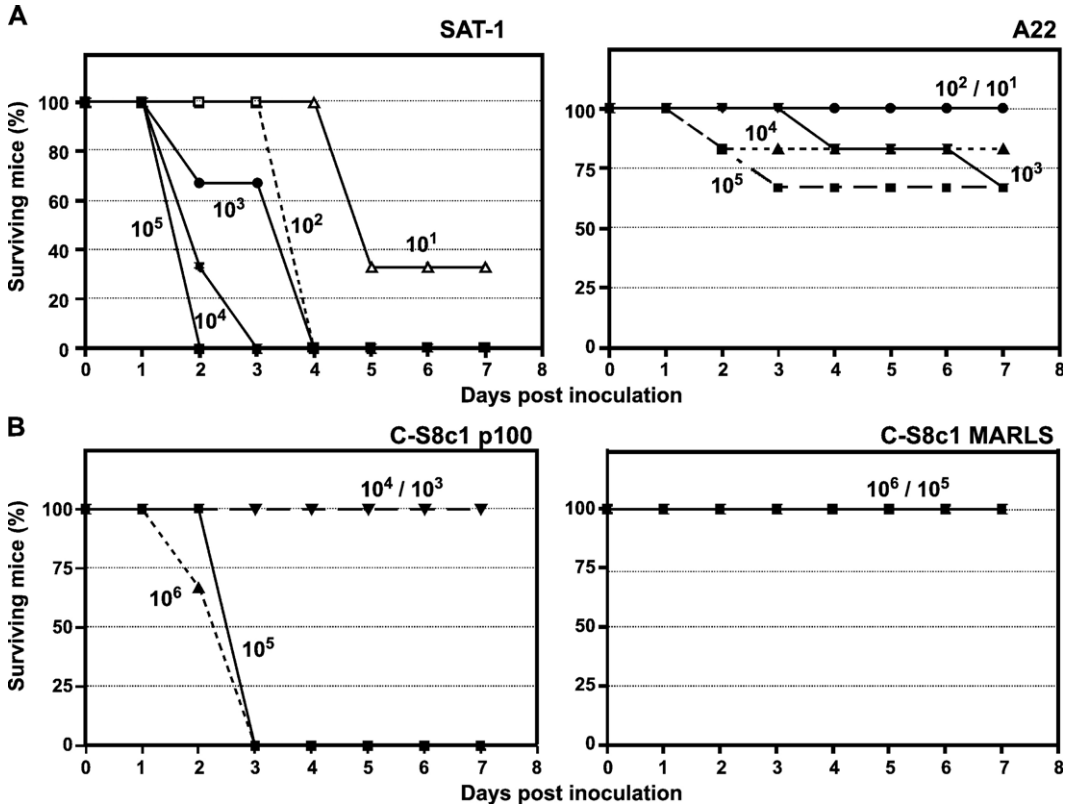

Fig. 4. Virulence of different FMDV serotypes and viral variants for C57BL/6 mice. Mice (8 weeks old, 6 per group) were subcutaneously inoculated in the footpad with 10-fold serial dilutions of each virus. (A) Survival curves of mice infected with FMDV SAT1 or A22. (B) Survival curves of mice infected with C-S8c1p100 or C-S8c1 MARLS. The number of PFUs administered to the mice is indicated on each survival curve. Values shown together indicate that the survival curves are superimposable. LD_{50} values are given in the text, and procedures are detailed in Materials and methods.

with A22 showed a remarkably different disease course (Fig. 4A). With the highest dose used to infect C57BL/6 mice (10^5 PFUs), only 33% of the mice died displaying similar symptoms to those observed after infection with FMDV C-S8c1 or SAT-1, within 24–48 h after infection. Thus, C57BL/6 mice showed different susceptibility to three FMDV serotypes, C, A, and SAT-1.

Additionally, we carried out experiments to determine the susceptibility of C57BL/6 to two variants of C-S8c1 generated by serial passage of C-S8c1 in BHK-21 cells (Charpentier et al., 1996; Martinez et al., 1997). We chose C-S8c1p100, the population that resulted after 100 passages of C-S8c1 in BHK-21 cells, that shows higher virulence for BHK-21 cells than C-S8c1 (Martinez et al., 1997). The second variant tested was MARLS, a clonal population selected from C-S8c1 passaged 200 times in BHK-21 cells, for its resistance to monoclonal antibody (Charpentier et al., 1996). C-S8c1 p100 showed an LD_{50} of 10^5 PFUs, approximately 10^3 times higher than the parental virus FMDV C-S8c1, while MARLS did not cause any death of C57BL/6 even at the highest dose used (10^6 PFUs) ($LD_{50} > 10^6$ PFUs) (Fig. 4B). These data suggest that C57BL/6 mice are adequate to detect differences in virulence among FMDV variants, and that multiple passages of FMDV C-S8c1 in BHK-21 cells resulted in an increase in replication for BHK-21 cells and a decrease of virulence for C57BL/6 mice.

FMDV infection of adult mice causes microscopic lesions that are characteristic of FMD

In order to better describe and understand the causes of lethal disease by FMDV in adult mice, histological analyses were performed on material of several organs taken from infected and uninfected C57BL/6 mice. Gross findings consisted on mild to severe edema of body cavities, mostly hydrothorax and hydropericardium, subcutaneous edema in the cervical area, enlarged heart, and reddish lungs. Histological examination of the heart showed signs of dilatation and mostly the right ventricle and the lung showed hyperemia, with a mild increase in the septum sizes (Fig. 5, panels A and B). A mild to severe lymphoid depletion was observed in the thymus, with an increase in the presence of "tangible-body" macrophages, showing the typical "starry-sky" appearance (Figs. 5A, B). Interestingly, the spleen and popliteal lymph nodes did not show significant changes. The footpad showed an increase in the degeneration of the cells of the stratum spinosum, mostly near the basal layer, showing intracellular edema and pyknotic nuclei. Moreover, intercellular edema was also found mostly in the stratum spinosum, and occasionally, microvesicles were found near the basal layer (Fig. 6). The types of lesions are typical of vesicular diseases, and are seen in FMDV infection of several natural hosts (see Discussion). The results suggest

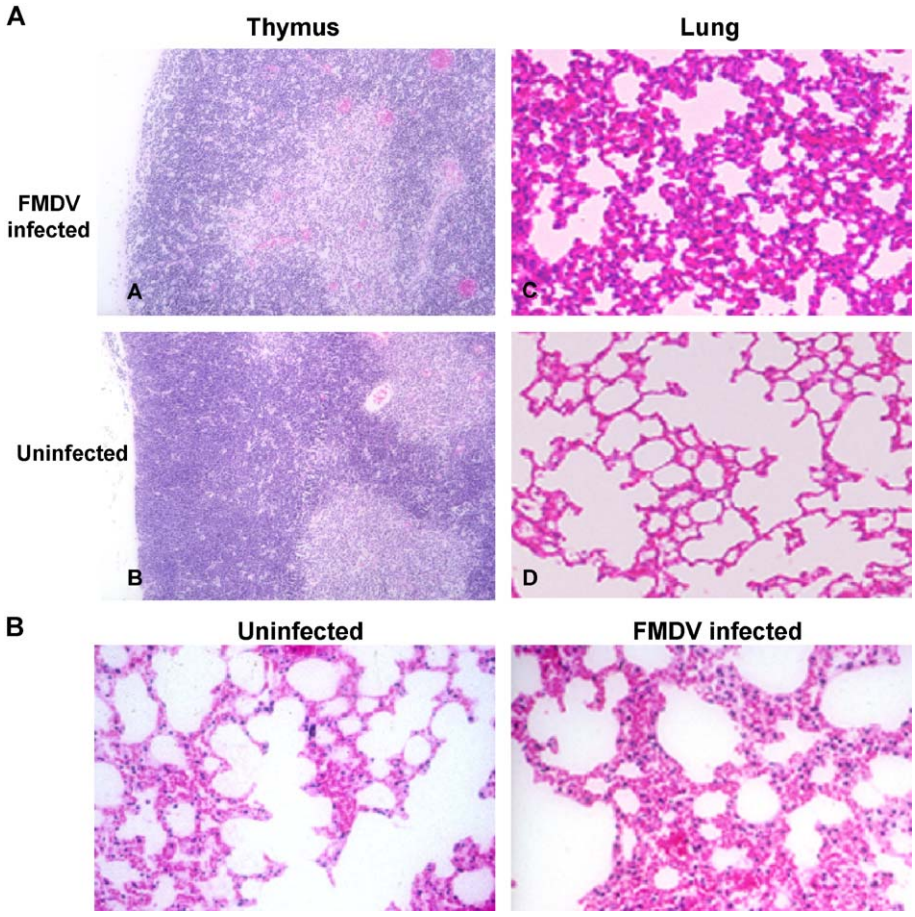

Fig. 5. Tissue sections of lung and thymus from FMDV-infected adult C57BL/6 mice. Mice were infected with 10^4 PFUs of FMDV subcutaneously in the LRFP. Tissues were harvested at 48 hpi. Hematoxylin and eosin staining are shown. Panel A, (A) Thymus from inoculated animal. Hyperemia and lymphoid depletion, mostly in the cortex, with the presence of numerous "tangible-body" macrophages that confers the typical "starry-sky" appearance, 100×. (B) Thymus from a non-inoculated, control animal, 100×. (C) Lung from an inoculated animal. Hyperemia and increased size of interalveolar septa, 100×. (D) Lung from a non-inoculated (naïve) control animal, 100×. Panel B. Higher magnification (200×) of additional tissue sections in which the increased size of the interalveolar septa in FMDV infected lungs is shown. Procedures are detailed in Materials and methods.

that the C57BL/6 mice respond to FMDV with microlesions typical of vesicular diseases that did not evolve to produce gross, macroscopic vesiculation.

In those infections that did not lead to death (oronasal infection) the histological examination of the lungs showed a marked increased in the septum sizes and hyperemia (data not shown). Examination of spleen showed a decreased in white pulp compared to uninfected control mice and, as a consequence, lost of marginal zone (data not shown). These findings indicate that following oronasal FMDV infection, C57BL/6 mice develop microlesions similar to those described in natural hosts (see Discussion).

Profound lymphopenia and lymphoid depletion during acute infection of mice with FMDV

Lymphocytes and white blood cells (WBC) were monitored in FMDV-infected C57BL/6 mice. Mice were inoculated with 10^4 PFUs of FMDV C-S8c1 or PBS in the LFRP. As compared to the mock-infected mice (PBS), at 24 hpi the FMDV infected mice had a significantly ($P < 0.001$) decreased lymphocytes and WBC count (Fig. 7A). The proportion of monocytes (M), polymorphonuclear leukocytes (PMN) and lymphocytes in blood of FMDV-infected mice were significantly altered when compared with control animals (Fig. 7A). The observed decrease in the percentage of lymphocytes and corresponding increase in the percentage of PMN suggest that the loss of WBC could be attributed to loss of lymphocytes relative to control animals.

The rapid removal of lymphocytes could be due either to cells being sequestered from the circulation and migration to lymphoid tissue, or to virus-induced apoptosis. To determine whether lymphocyte depletion occurred also in lymphoid tissue, spleen lymphocytes were analyzed by flow cytometry. C57BL/6 mice were inoculated in the LRFP with 10^4 PFU of FMDV C-S8c1. At 24 hpi, spleens from FMDV-infected and mock-infected mice were harvested, showing a profound increase in the spleen size as compared to the mock-infected mice. Counting of cells showed a 4-fold increase in the number of splenocytes (Fig. 7B). Double

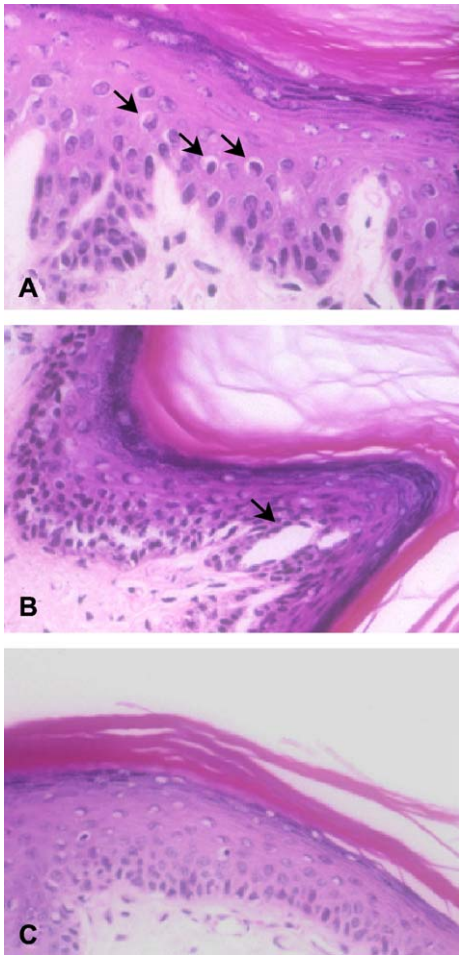

Fig. 6. Tissues sections of footpad from C57BL/6 mice inoculated with FMDV. Mice were infected with 10^4 PFUs of FMDV subcutaneously in the LRFP. Tissues were harvested at 48 h post-inoculation. Hematoxylin and eosin staining are shown. (A) Inoculated animal. Intracellular spongiosis, vacuolization and pyknosis of cells from the spinous layer (arrows), 630×. (B) Inoculated animal. Intercellular oedema in the spinous layer and formation of a suprabasilar vesicle (arrow), 400×. (C) Same tissue of a non-inoculated, control animal, 400×. Procedures are detailed in Materials and methods.

staining to detect CD4 and CD8 lymphocytes identified a significant loss of $CD4^+$ and $CD8^+$ T cells in spleen of FMDV infected mice (Fig. 7B). The induction of this loss of T cells applied to both T cell subsets equally, with no statistically significant difference between $CD4^+$ and $CD8^+$ T cell loss in infected mice. These data indicate that lymphocyte depletion also occurred in lymphoid organs, suggesting that the lymphocytes may be dying as a result of the viral infection.

To identify if lymphocytes were infected by FMDV, peripheral blood lymphocytes (PBLs) were purified from peripheral blood from FMDV C-S8c1-infected and naive mice. Cells were labeled with specific monoclonal antibodies to CD8, CD4, and B220 surface markers couple to a fluorochrome probe. This was complemented with the use of an antibody to FMDV 3D or FMDV VP1 to differentiate between viral replication and bound virus, coupled to a different and distinct fluorochrome, thereby allowing detection of viral antigen in the specifically marked cell type. FMDV C-S8c1 primarily infected $CD8^+$ and $B220^+$ cells with only minimal to negligible infection of $CD4^+$ T cells (Fig. 7C). These data indicate that $CD8^+$ T cells and $B220^+$ lymphocytes were infected by FMDV, possibly contributing to the T cell lymphocyte depletion observed. However, the fact that $CD4^+$ T cells were not infected suggests that other mechanism that direct viral infection could be implicated in the lymphocyte depletion in FMDV-infected mice.

Immunized mice are completely protected against a lethal FMDV challenge

The clinical course, distribution of virus in the body, and pathological findings indicated that FMDV caused a systemic lethal infection in adult, immunocompetent C57BL/6 mice. To provide further proof that FMDV was the causative agent of the disease in these animals and that mice could respond to FMDV vaccine antigen, we performed vaccination protection experiments. Adult C57BL/6 mice were immunized with chemically inactivated FMDV C-S8c1 vaccine (Bahnemann, 1975). The vaccine was administered by two consecutive intraperitoneal injections of the equivalent to 10^6 PFU at intervals of 2 weeks. Following each injection, antibody titers were determined by ELISA. All immunized animals, but not control animals inoculated with PBS, developed an antibody response, indicating successful immunization (Fig. 8A). Subsequently, 2 weeks after the last immunization, immunized and control animals were challenged in the left rear footpad with 10^4 PFUs of FMDV C-S8c1. While all control animals died, 100% of the immunized animals were protected against a lethal challenge (Fig. 8B). This result indicates that protective immunity was achieved and supports the notion that FMDV was the causative agent of disease and death of adult C57BL/6 mice.

Discussion

This new mouse model for the study of FMDV infections has unique features that open the possibility to study in the same host-virus system susceptibility and resistance to lethal infection, immunobiology of infection and vaccine efficacy, in ways that are not approachable with the natural hosts, or other animal model systems for FMDV. Unlike other animal systems, the advantage of the mouse model is not only the availability of a wide variety of reagents, but also the use of an inbred mouse strain with a uniform genetic background. Furthermore, the potential of using genetically altered mice to study many aspects of the immune response to the virus opens new fields of research in the analysis of the immune response to FMDV infection in vivo.

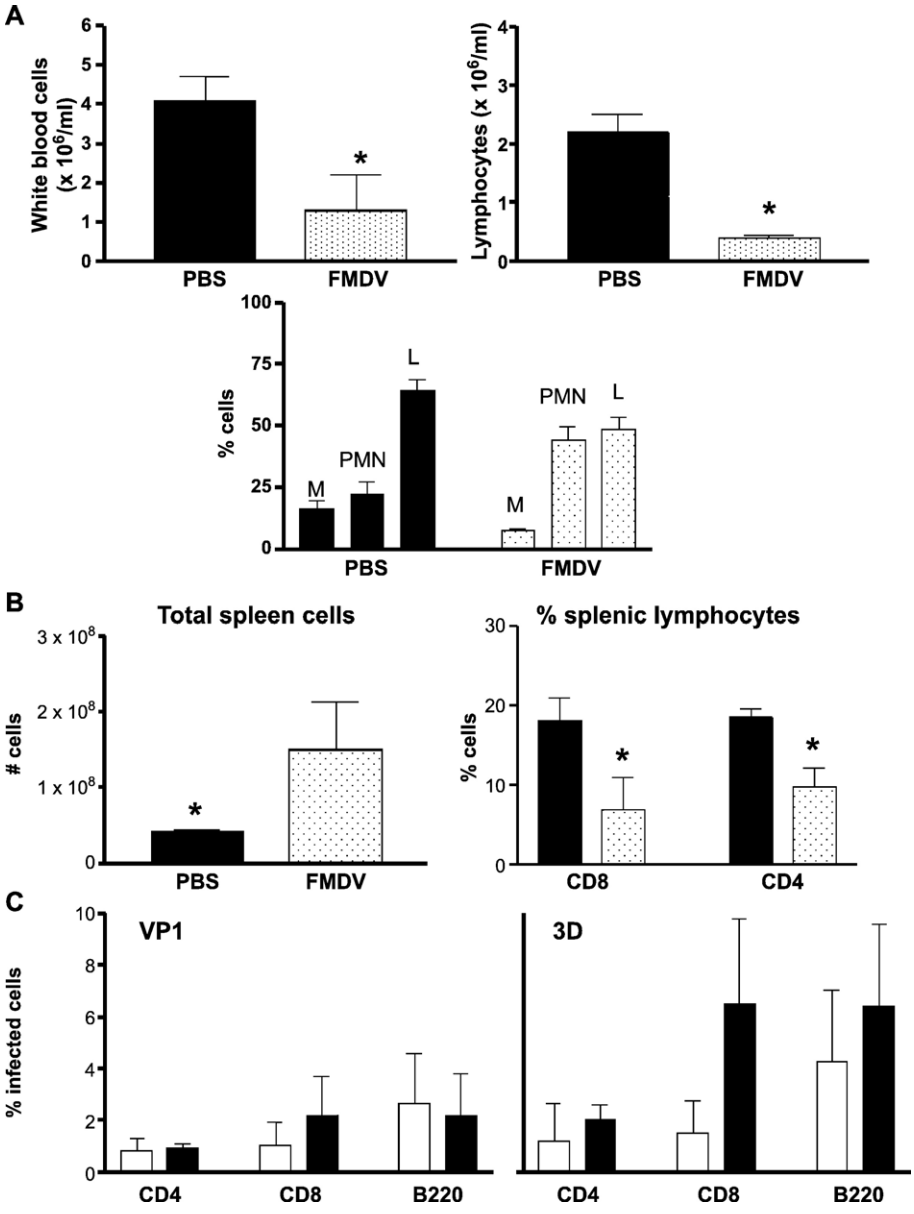

Fig. 7. Lymphopenia and T cell depletion in FMDV infected C57BL/6 mice. Mice were inoculated in the LRFP with 10^4 PFU of FMDV C-S8c1 and sacrificed at 24 hpi. (A) Blood was collected and the WBC and lymphocytes counts were determined as described in Materials and methods. Leukocytes are indicated as follow: mononuclear cells (M), polymorphonuclear cells (PMN) and lymphocytes (L). Values were compared to control samples obtained from mock-infected mice. Data are shown as the average of results ±SD, with $n = 8$ for the infected and $n = 5$ for the mock-infected mice. Asterisks denote a statistically significant reduction in infected when compared to mock-infected mice (Student's t test, $P < 0.05$). (B) Spleens were harvested from FMDV-infected or mock-infected mice at 24 hpi and the total splenocyte number was determined. Flow cytometric analyses were performed using antibodies directed against CD4 and CD8α to determine the number of splenic T cells. A minimum of 50,000 events was gated, and data were plotted as the percentage of positive events ±SD for each population of T cells. Asterisks denote a statistically significant reduction in infected when compared to mock-infected mice (Student's t test, $P < 0.05$). (C) Comparative levels of infectivity in T cells caused by FMDV C-S8c1. In each experiment, four adult C57BL/6 mice were injected in the LRFP with 10^4 PFU of virus, and were sacrificed at 12 (white bars) and 24 (black bars) hpi (see Materials and methods). PBLs were isolated from total blood and were labeled with cell-specific monoclonal antibodies to CD4, CD8, and B220. Subsequent intracellular staining using specific antibody to FMDV VP1 (antibody 2E5) or 3D (antibody 3H11) was performed, followed by FACS. The graph represents percentages of cells in each subset infected with FMDV C-S8c1.

Infection of adult mice with FMDV was dependent on a number of variables, including dose, route of inoculation and genetic background of the mouse, and the virus. SJL/J and Swiss mice were less susceptible to FMDV C-S8c1 than C57BL/6 and BALB/c mice, suggesting that mouse susceptibility to FMDV may be linked to genetic factors. It has been amply documented that the route of inoculation is another important determinant of the outcome of viral infections (Bray et al., 1998; Houtman and Fleming, 1996; Rai et al., 1996; Turner and Moyer, 2002; Wichmann et al., 2002). Subcutaneous (footpad) and ip inoculations of C57BL/6 mice with FMDV were lethal, although ip

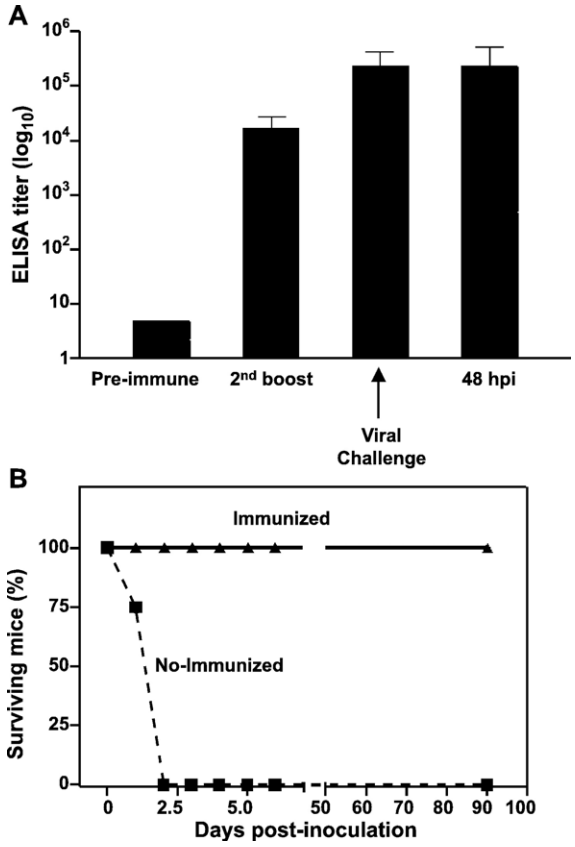

Fig. 8. Protection of vaccinated C57BL/6 mice against a lethal FMDV challenge. Mice (8 weeks old, 18 per group) were immunized twice by intraperitoneal administration of an inactivated virus vaccine, prepared as described in Materials and methods. (A) Sera from mice previous to immunization (pre-immune), day of the second booster (2nd boost), day of viral challenge (10^4 PFUs of FMDV C-S8c1 in the LFRP) and 48 hpi were used to determine antibody (Ab) titers against FMDV by ELISA, as described in Materials and methods. In contrast to the controls animals (not shown), all immunized mice developed an antibody response, indicating successful immunization. (B) While all control animals died, protective immunity was achieved in 100% of the immunized mice.

inoculation caused death faster. This result is not surprising since sc inoculation generally produces a slower replication and dissemination of the virus, which may allow sufficient time for the host to activate clearance mechanisms. An important difference between the sc and ip routes of infection that may explain differences in outcome is the antigen-presenting cell populations involved in initiating the immune response. Sc infections involve peripheral dendritic cells (DC) populations, such as Langerhans cells or lymphoid interdigitating DC as the initial presenting cells, whereas ip infection probably results in presentation to T cells via peritoneal macrophages and macrophage-derived DC. Differences in viral kinetics between the two routes of infection may, therefore, be due to differences in the antigen-presenting cell populations or to the cytokine milieu associated with peripheral DC and peritoneal macrophages (Banchereau et al., 2000; Pulendran et al., 2001). Oronasal inoculation, however, did not lead to death in C57BL/6 mice although the mice developed microscopic lesions. The resistance shown by C57BL/6 mice to FMDV when inoculated by the oronasal route compares to the relative resistance that swine show to infection with airborne FMDV suggested that a dose of more than 800 TCID50 is required to cause infection and typical disease (Alexandersen and Donaldson, 2002). For all that, it could be possible that the virus spread slower after oronasal inoculation and the immune system control the infection before the virus causes a systemic infection. Experiments are currently being done to understand the different outcome of FMDV infection in mice depending of the inoculation route.

The classical description of viral pathogenesis holds that viruses first replicate at the site of inoculation, followed by spread to the draining lymph node (Mims et al., 1995; Nathanson, 1980). The presence of FMDV in the draining lymph node (PLN) was documented at 24 hpi, after viremia had been established (Fig. 3), suggesting that FMDV may not require to infect DCs to move to the draining lymph node. In other viral infections, the detection of early virus in the draining lymph nodes has been associated with infection of Langerhans cells in the footpad followed by migration of these cells to the draining lymph node (Hertel et al., 2003; MacDonald and Johnston, 2000).

Histological analysis of FMDV-infected mice at 48 hpi shows a similarity between lesions found in the footpad and those described during infection of the natural hosts (reviewed in (Meyer and Knudsen, 2001). The stratum spinosum shows a degeneration of the body cells that corresponds with pyknotic nuclei. The appearance of microvesicles near the basal layer suggests that FMDV induces the formation of vesicles in the footpad, a main characteristic of the disease in the natural host. These observations imply that although the development of epidermal vesicles in C57BL/6 mice infected with FMDV was not apparent in a macroscopic analysis, the events preceding vesiculation were observed. The lesions which FMDV produces in adult C57BL/6 mice are quite different, in many respects, from those described for infection of young mice (Platt, 1956). In 7-day-old mice, FMDV has a marked myopathic affinity, and produces extensive destruction of striated muscles, while in the mature animal, it elicits the more typical epithelial reaction. Similarly, in cattle the virus may produce rapid death in calves by a necrotizing effect on striated muscle tissue, while in the fully grown animal the lesions are predominantly epithelial.

A profound lymphopenia has been described during the acute infection of swine with FMDV (Bautista et al., 2003). In our mouse model system, we have shown that there is a significant lymphopenia and lymphoid depletion in lymphoid organs at 24 hpi that affects all T cell subsets, creating a transient immunosuppression. The analysis of viral replication in lymphocyte subsets shows that FMDV replicates in $CD8^+$ and $B220^+$ cells. As the percentage of

infected cells is not higher than 10%, we cannot exclude an induction of apoptosis in lymphocytes by bystander effect. In fact, the lack of FMDV infection in $CD4^+$ cells strongly suggests that other factors different to direct viral infection are playing a role in cell death. One possibility could be activated monocytes expressing several soluble mediators of apoptosis, including tumor necrosis factor alpha (TNF-), CD30L, FasL, 4-1BBL, and TNF-related apoptosis-inducing ligand (TRAIL) (Ashkenazi et al., 1999; Dockrell et al., 1998; Pauza et al., 2000; Yang et al., 2003). Any of these factors could induce cell death in lymphocyte populations in FMDV-infected mice. The role of some of these molecules in the induction of lymphocyte death is the focus of current investigations in our laboratory. Additionally, the effect of secreted soluble factors by monocytes/dendritic cells probably plays a significant role in the ability of the virus to spread systemically in the mouse and to cause death. The significance and contribution of the lesions described to death is difficult to assess since we have not found any highly destructive effect upon any particular tissue analyzed. A cytokine-mediated lethality during viral infection cannot be excluded (Kapcala et al., 1995).

The differences observed among FMDV serotypes in disease course after inoculation of C57BL/6 mice (Fig. 4A) are striking. Differences in pathogenicity associated to serotypes have been described for other viral infections (Tyler and Fields, 1990), but it is not well known for FMDV. In addition, two viruses, C-S8c1p100 and C-S8c1MARLS, which are closely related both evolutionarily and antigenically(Charpentier et al., 1996) differ in their pathogenic properties for C57BL/6 mice. This may allow the mapping of virulence determinants and may help defining components of a protective response against FMDV.

The protection mediated by inactivated whole FMDV vaccine in C57BL/6 mice infected with a lethal dose of FMDV C-S8c1 was complete, in that 100% of the animals did not show any symptom or death associated with the infection. This result strongly supports the involvement of FMDV in the pathological processes described in this work and, moreover, suggests that the mouse may be an adequate model system to compare vaccine candidates. However, it remains to be seen whether with other vaccine formulations, the capacity to protect mice parallels the capacity to protect natural hosts.

In conclusion, we have developed an adult mouse model to study FMDV infections. The virus causes a lethal systemic infection in several inbred strains of adult, immunocompetent laboratory mice. This is the first time that death of adult mice is associated with FMDV infection. Even though mice are not known to be natural hosts for FMDV, by virtue of the detailed knowledge of their genetics and immunological mechanisms, this host will provide data that may contribute to guide in experiments for the understanding of the mechanisms of FMDV infection in natural hosts.

Materials and methods

Animals

C57BL/6, BALB/C, SJL/J and Swiss mice were purchased from Harlan Interfauna Iberica, S.L. All mice used were matched for sex and age (females, 8–10 weeks). Mice were maintained under pathogen-free conditions and allowed to acclimatize to the biosafety level 3 (BSL3) animal facility at the Centro de Investigación en Sanidad Animal, INIA, Madrid, for 1 week before use in our experiments. All experiments with live animals were performed under the guidelines of the European Community (86/609) and were approved by the site ethical review committee.

Viruses and cells

Viruses were growth in baby hamster kidney cells (BHK) as described (Domingo et al., 1980). Procedures for infection of BHK-21 cell monolayers with FMDV in liquid medium and for plaque assays in semisolid agar medium have been described previously (Baranowski et al., 1998; Domingo et al., 1980; Sobrino et al., 1983). The FMDV C-S8c1 is a plaque-purified derivative of natural isolate C_1-Sta Pau-Spain 70, a representative of the European subtype C_1 FMDVs (Sobrino et al., 1983). FMDV C-S8c1p100 is the virus C-S8c1 propagated 100 times in cell culture (Martinez et al., 1997). FMDV MARLS is a monoclonal antibody escape mutant obtained from FMDV C-S8c1p213 (C-S8c1 passaged 213 times in BHK-21 cells) (Baranowski et al., 1998; Charpentier et al., 1996). FMDV A22 and SAT1 were kindly provided by E. Blanco, Centro de Investigación en Sanidad Animal, INIA, Madrid.

Animal infection and preparation of samples

Mice were infected with FMDV either subcutaneously (sc) in the left rear footpad (LRFP) (50 μl) or intraperitoneally (ip) (100 μl) with different amount of viruses. The oronasal inoculation was done in a volume of 50 μl. Mice were examined for clinical symptoms twice daily. LD_{50} were calculated by the method of Reed and Muench (1938), after inoculation of mice with 10-fold serial dilutions of virus. At varying times post-infection, several mice were euthanized and organs (liver, lung, spleen, heart, kidney, pancreas, brain, popliteal lymph node [PLN], and thymus) were harvested and weighed. Half of the tissues were used for histological analyses. The other half was homogenized using an Omni tissue homogenizer (Omni International). Serum was isolated from whole blood that was obtained from the intraorbital sinus. The amount of infectious virus was measured by plaque assay on BHK cells as described (Domingo et al., 1980).

Hematologic analysis

Blood was collected from the intraorbital sinus in 5 μM EDTA at different times post-inoculation. The anticoagulated blood sample was thoroughly mixed and analyzed on an AcT Hematology analyzer (Beckman Coulter, Hialeah, FL) following manufacturer's directions. The percentages of leukocyte populations (monocytes, polymorphonuclear leukocytes and lymphocytes) were determined by staining with May–Grunwald–Giemse and counting at least 300 cells per sample.

Histopathology

Samples from different tissues and organs were taken and fixed in 10% buffered formalin (pH 7.2) for histopathological studies. After fixation, samples were dehydrated through a graded series of alcohol to xylol and embedded in paraffin wax. Sections of 4-ìm-thick were cut and stained with hematoxylin and eosin (H&E) for histopathological analysis.

Isolation of spleen cells, peripheral blood lymphocytes (PBL) and flow cytometric analysis

Spleens were obtained from C57BL/6 mice infected with FMDV C-S8c1. Single cells were then obtained by mechanical disruption. Cells were pelleted by centrifugation and resuspended in staining buffer (PBS containing 2% (vol/vol) fetal bovine serum and 0.2% (wt/vol) NaN_3) for flow cytometry. PBLs were isolated from peripheral blood of C57BL/6 mice infected with FMDV C-S8c1. Briefly, fresh heparinized peripheral blood was mixed with an equal volume of phosphate-buffered saline (PBS). Ficoll-Hypaque (density 1.007 g/litter) was layered underneath the blood/PBS mixture and centrifuged 30 min at 900 × *g*. The mononuclear cell layer was transferred to another tube, washed, and counted with Trypan Blue to determine viability. To analyze the expression of cell surface molecules, we used monospecific antibodies, fluorochrome dyes, and flow cytometry, as described (Sevilla et al., 2000). The antibodies used were rat anti-mouse CD8α-PerCp, rat anti-mouse CD4-FITC and anti-rat B220-PE (all from BD Pharmingen, San Jose, CA, USA). After staining, cells were fixed in PBS/1% fetal bovine serum/4% PFA (wt/vol). Affinity-purified antibody to FMDV-3D (3H11) was conjugated to Alexa-488 according to the protocol recommended by the manufacturer (Molecular Probes) and used for intracellular detection of FMDV 3D. Ascites of the antibody FMDV-VP1 (2E5) was used to detect FMDV VP1. An anti-mouse conjugated to Alexa-488 was used as secondary antibody for FMDV-VP1. Cells were acquired using a FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA). Dead cells were excluded on the basis of forward and side light scatter. Data were analyzed with FlowJo (Tree Star, San Francisco, CA, USA) and CellQuest software (Becton Dickinson).

Antibody response

Anti-FMDV antibodies (Ab) were detected by ELISA as described (Novella et al., 1993). Briefly, 96 well polyvinylchloride (PVC) microplates (Flow Laboratories) were coated at 4 °C overnight with purified FMDV C-S8c1 (100 ng of VP1). Mouse serum was diluted into PBS/3% ovalbumin and analyzed for the presence of IgG antibodies using horseradish peroxidase-conjugated Ig antibodies (BioRad) to develop the reaction. *O*-Phenylenediamine-H_2O_2 (Sigma) was used as a peroxidase substrate and the absorbance was read at 490 nm in a Microplate Reader (Labsystems Multiskan MS). Titers are reported as the reciprocal of the highest dilution to give an OD_{A490} of >0.200. Uninfected control mouse sera were uniformly negative at the lowest dilution tested, 1:10.

Mice immunizations

Groups of 18 C57BL/6 mice were immunized by two consecutive ip injections of either gradient-purified binary ethylenimine (BEI)-inactivated FMDV C-S8c1 preparation (Bahnemann, 1975) or phosphate-buffered saline (PBS) (controls) emulsified in Freund's incomplete adjuvant, administered 2 weeks apart. Mice were sc inoculated in the footpad with 10^4 PFUs of FMDV C-S8c1 (lethal dose) 2 weeks after the last immunization. Mice were bled before each immunization and virus challenge. Sera were tested for FMDV antibodies by ELISA as described above.

Statistical analyses

Data handling, analysis and graphic representation was performed using Prism 2.01 (GraphPad Software, San Diego, CA). Statistical differences were determined using a Student's *t* test ($P < 0.05$).

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