Duration of the foot-and-mouth disease virus antibody response in mice is closely related to the presence of antigen-specific presenting cells

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Natural and experimental hosts infected with foot-and-mouth disease virus (FMDV) develop a long-lasting immune response that is closely related to the presence of anti-FMDV antibodies (Ab). We show here that spleen cells from animals which had been infected 3 or more months previously induced an anti-FMDV-Ab response in untreated animals which lasted more than 210 days after cell transfer. Persistence of infectious virus was excluded since virus isolation or detection of the viral genome by PCR in donor splenocytes were consistently negative. The role of antigen presentation (AP) in this phenomenon was studied in vivo by using irradiated splenocytes from virus-sensitized donor mice. Although these irradiated cells were unable to induce anti-FMDV-Ab in normal or irradiated recipient mice, they elicited a strong secondary reaction in FMDV-pre-sensitized recipients. The presence of AP cells (APC) presenting FMDV epitopes (FMDV/APC) was also analysed in mice sensitized to FMDV in different ways. A close correlation between FMDV/APC and the presence of anti-FMDV-Ab was found in infected mice as well as in mice immunized with different doses of inactivated virus, with or without adjuvants. Experiments in vivo and in vitro showed that the APC activity can be specifically blocked with either anti-MHC class II monoclonal antibody or anti-FMDV antiserum, and is dependent on the presence of T cell function. These results strongly suggest that persistent FMDV/APC are responsible for the existence and maintenance of an anti-virus immune response regardless of the immunization method used.

Introduction

Foot-and-mouth disease virus (FMDV) is the causative agent of a highly contagious disease that affects biungulate species (Cottral, 1975). Although extensive information dealing with the antigenic structure of the virus and its role in the induction of specific antibodies (SA) is available, little is known about the immune mechanisms involved in the maintenance of the induced immune response. Natural infection, or immunization with inactivated virus, induces SA which effectively protect the host against a challenge with virulent virus (Berinstein et al., 1993; Brown, 1992; Cottral, 1975; Lopez et al., 1990; Morgan et al., 1980; Piatti et al., 1991). The immunity elicited by infection, both in cattle and mice, provides protection for a very long period and appears to correlate with the presence of circulating SA (Borca et al., 1986; Cunliffe, 1964; Fernandez et al., 1986). On the other hand, immunization with inactivated virus vaccines in natural hosts is reported to result in a shorter-lasting SA response and protection (Morgan et al., 1980) although some reports indicate that long-lasting immune responses could be achieved by repeated vaccination (Terpstra et al., 1990).

Interestingly, the duration of the immune response in mice immunized with purified inactivated virus (PIV) is directly related to the antigenic mass given, and it can last for life when high doses of virus are used (Berinstein et al., 1993; Lopez et al., 1990; Piatti et al., 1991). Although these differences in the responses elicited by inactivated or infectious virus have been frequently reported (Morgan et al., 1980), their actual immunological basis remains largely unknown. An understanding of this phenomenon is of importance for the formulation of vaccines of prolonged efficacy.

The results presented here show that in mice, the
maintenance of an immune response to either infectious virus, or PIV is closely related to the presence of virus-specific antigen-presenting cells (APC).

Methods

Mice. Sixty to ninety-day-old male or newborn BALB/c mice from our colony were used throughout the study: $m_{1/2}$ mice and their littersmates were kindly provided by the Comisión Nacional de Energía Atómica de la República Argentina.

Virus. Mice were infected by intraperitoneal (i.p.) inoculation of 0.5 ml PBS containing 10^4 suckling-mouse 50% lethal doses (SMLD$_{50}$) of FMDV subtype O1Campos (O1C) (Borca et al., 1986; Fernandez et al., 1986). PIV was obtained by one passage of the stock virus in BHK-21 cells. Virus in supernatants was inactivated with binary ethyleneimine (BEI), purified in a 10–30% sucrose gradient and quantified as described elsewhere (Berinstein et al., 1993).

Irradiation procedure. Irradiation was conducted at the Comisión Nacional de Energía Atómica de la República Argentina, with gamma-ray equipment. Mice received whole-body irradiation with a dose of 5.5 gray (Gy), whereas splenocytes were irradiated in vitro with a dose of 15 Gy.

Antibody response. Anti-FMDV antibodies (Ab) were detected by ELISA as described by Perez-Filgueira et al. (1995), with slight modifications. Briefly, Nunc immunoplate I maxisorp ELISA plates (Dynatech) were coated at 4 °C overnight with a 1:500 dilution of rabbit anti-FMDV O1C antisum in carbonate–bicarbonate buffer, pH 9.6. Supernatant of BHK-21 FMDV O1C-infected cells cultures was then added to the plate and incubated for 1 h at 4 °C. Blocking and subsequent steps were performed with PBS containing 3% horse serum and 0.025% Tween 20. Mouse serum to be tested was diluted 1:4 in blocking buffer, added to the plate and incubated for 45 min at 37 °C. Peroxidase-labelled anti-mouse Ig antibodies (Dakopats) were used to develop the reaction. O-Phenylenediamine–H$_2$O$_2$ was used as a peroxidase substrate and the A was read at 490 nm in a MR 5000 Microplate Reader (Dynatech).

Anti-ovalbumin (OVA) antibodies were also measured by ELISA. Immulon II plates were coated at 4 °C overnight with a solution of OVA (1 µg/ml) in carbonate buffer. The subsequent steps were similar to those described for the detection of anti-FMDV-Ab. Anti-FMDV and anti-OVA titres were expressed as the log$_{10}$ of the inverse of the highest serum dilution that at least duplicates the A obtained with a negative serum.

Repopulation assays. Spleen cell suspensions were obtained as described in detail elsewhere (Berinstein et al., 1993; Borca et al., 1986). Briefly, unfractionated splenocytes were obtained by gentle homogenization of spleens in 199 medium containing 5% FCS and 1% penicillin–streptomycin (complete medium), followed by osmotic lysis of the erythrocytes with 0.75% Tris–NH$_4$Cl (pH 7.2). Cell suspensions, 1.5 × 10^6 per mouse unless otherwise indicated, were inoculated i.p. into recipient mice; the adoptive transfer experiment, performed using smaller amounts of cells (10^5), induced inconsistent antibody responses in recipient mice. Cell transfers were usually performed between 60–90 days post-infection. Non-adherent cells were obtained by incubating spleen cells in glass Petri dishes at 37 °C for 1 h in 5% CO$_2$. Purified B cells were obtained from non-adherent cells by treatment with anti-Thy 1.2 monoclonal antibodies (MAbs) plus guinea-pig complement.

Purified T cells were obtained by passing total spleen cells through nylon columns (Mishel et al., 1980) and subsequent treatment with anti-lg polyclonal antibodies (obtained in rabbits) plus guinea-pig complement. The purity of the preparations was assessed by direct immuno-fluorescence using isothiocyanate-labelled anti-Thy 1.2 and anti-B220 MAbs. Only preparations showing a purity over 94% were further used.

Antigen presentation (AP) assay. Donor splenocytes obtained at the desired time post-infection were in vitro-irradiated (15 Gy) and transferred i.p. 24 h later to the recipient mice. Recipients were pre-immunized 30–45 days before the cell transfer with 0.5 µg PIV in PBS. Before being used as recipients, mice were tested for the presence of anti-FMDV-Ab. Only mice showing ELISA titres of ≥ 6 were used. The development of an anti-FMDV anamnestic response was tested in the recipients 9 days after cell transfer.

Inhibition of AP. Spleen cells (5 × 10^6 cells per well), from mice infected with FMDV or immunized with 10 µg of OVA in incomplete Freund's adjuvant (IFA) 90 days earlier, were pre-incubated with different blocking antibodies or complete medium alone for 24 h in a 96-well plate at 37 °C in 5% CO$_2$. The cells were then washed three times in complete medium and incubated for a further 6 days at 37 °C in 5% CO$_2$. Presence of anti-FMDV or OVA antibody was tested for in the supernatant of the cell culture by ELISA at the end of the experiment. Anti-FMDV and anti-OVA blocking antibodies were produced in our laboratory by immunizing rabbits with PIV or OVA (Sigma) in IFA. Anti-I$^a$ MHC class II antigen MAb was purchased (Sigma); MAbs reacting with L3T4, B220 and Thy 1.2 were obtained from ATCC (TIB 207, 140 and 99, respectively).

Vaccine formulation. Vaccines were prepared with the desired concentration of PIV, in PBS or emulsified in IFA. Immunomodulators used in this work were Avridine (AVR) and the water-soluble fraction (WSF) of the cell wall of Mycobacterium spp. (Berinstein et al., 1993). Vaccines using immunomodulators contained 1 µg of PIV per dose.

Results

Adoptive transfer of the anti-FMDV immune response to naive recipients

To understand the basis of the persistent antibody response induced by infection, immunocompetent cells from infected mice were adoptively transferred to naive recipient mice. The mice that received spleen cells from mice infected up to 180 days earlier developed a strong anti-FMDV-Ab response which was detectable at 30 days after cell transfer and lasted for the duration of the 7 month experimental period (Fig. 1a). The induction of this immune response could be due to (i) the transfer of infectious virus from the donor to the recipient, (ii) the transfer of cells actively producing antibodies or (iii) the transfer of APC bearing FMDV epitopes.

The presence of infectious virus in the transferred splenocytes was ruled out by (a) inoculating them into suckling mice, and (b) coculturing them with primary bovine thyroid cell cultures, which are extremely sensitive to FMDV (Lopez et al., 1990). By neither of these two methods was it possible to demonstrate the presence of infectious virus (data not shown). In addition, RT–PCR analysis of the transferred spleen cells (2 × 10^6 cells), using a pair of 20-mer oligonucleotide primers corresponding to positions 598–958 of the FMDV polymerase gene, followed by Southern blot analysis of the amplified
To study the mechanisms involved in the induction of the immune response in the recipient animals, a system for detecting the presence of FMDV epitopes was developed. Splenocytes from infected donors, which were irradiated 24 h before the cell transfer, were shown to induce a strong anamnestic immune response in recipient mice which had been pre-immunized with 0.5 µg of PIV 30–45 days earlier (Table 1, Group 7). The irradiation immunosuppressed the transferred splenocytes (compare the effect of the irradiation on the function of cells in Group 4 with that of the mock-irradiated cells in Group 3), so any anti-FMDV-Ab detected in the new host must have been produced by its own immune system. The presence of such an anamnestic response was absolutely dependent on the FMDV pre-sensitization, since no response could be induced if the recipients were not pre-immunized with FMDV (compare the immune response elicited by the APC in recipients which were pre-sensitized, Group 7, with that elicited in mock pre-immunized recipients, Group 2). It should be noted that at the time of the cell transfer, the recipient had already became negative for antibodies to FMDV (Group 1). Importantly, the irradiated donor-cell population was itself completely unable to mount an anti-FMDV-Ab response (Group 2), even when the recipient host was also irradiated (Group 4), or when elicitation of a secondary immune response was attempted by infecting the recipient mice (Group 6). Therefore, the presence of FMDV antigens provided by the irradiated donor-cell population actually induced an anamnestic immune response from the pre-sensitized recipient immune system.

The presence of APC presenting FMDV epitopes (FMDV/APC) was analysed in spleens of naïve recipient mice (naïve first recipient) which had developed an anti-FMDV response after receiving splenocytes from donors infected 135 days earlier. It was possible to detect a strong APC activity in those naïve recipients that had received splenocytes from infected donors 90 days earlier (Fig. 2). Since these naïve recipient mice were never exposed to FMDV antigens this result indicates the existence of cells actively presenting FMDV epitopes among the splenocytes originally received from the infected donors. Additionally, when the same cells (those used for testing APC activity) were transferred, without irradiation, to a new generation of naïve recipient mice, they completely failed to induce an anti-FMDV response in the new hosts (Fig. 2). This result suggests that insufficient APC could be delivered to the second generation of naïve recipients, and also supports the fact that no infectious virus is involved in the induction of the transfer of the immune response.

**Correlation between the presence of FMDV/APC in immune mice and the quality of their anti-FMDV immune response**

The presence of FMDV/APC was analysed in mice that were sensitized to the virus in different ways. Initially, the APC activity in mice that had been infected for a variable period of time was studied. Splenocytes as well as purified B lymphocytes from mice infected 45, 90 or 365 days earlier were very

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**Detection of FMDV/APC in the transferred spleen cells**

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Table 1. Development of the antigen presentation assay

<table>
<thead>
<tr>
<th>Group</th>
<th>Donor treatment*</th>
<th>Recipient treatment†</th>
<th>Anti-FMDV titres‡</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>Pre-sensitized</td>
<td>0·3</td>
<td>0·00</td>
</tr>
<tr>
<td>2</td>
<td>Infected and γ-ray</td>
<td>None</td>
<td>0·60</td>
<td>0·20</td>
</tr>
<tr>
<td>3</td>
<td>Infected</td>
<td>γ-ray</td>
<td>2·70</td>
<td>0·35</td>
</tr>
<tr>
<td>4</td>
<td>Infected and γ-ray</td>
<td>γ-ray</td>
<td>0·45</td>
<td>0·15</td>
</tr>
<tr>
<td>5</td>
<td>Infected</td>
<td>γ-ray and infected</td>
<td>3·50</td>
<td>0·00</td>
</tr>
<tr>
<td>6</td>
<td>Infected and γ-ray</td>
<td>γ-ray and infected</td>
<td>0·30</td>
<td>0·00</td>
</tr>
<tr>
<td>7</td>
<td>Infected</td>
<td>Pre-sensitized</td>
<td>2·60</td>
<td>0·30</td>
</tr>
</tbody>
</table>

* Splenocytes from donor mice were irradiated in vitro with 15 Gy 24 h before cell transfer.
† Cells (1·5 x 10⁶ donor splenocytes) were transferred to recipient mice. FMDV pre-sensitization was performed, with a single i.p. dose of 0·5 µg purified inactivated FMDV O1C in PBS, 30–45 days before cell transfer. Receptors were irradiated with 5·5 Gy 24 h before cell transfer.
‡ Anti-FMDV-Ab titres were measured by ELISA in the receptors 9 days after cell transfer. Titres are the mean data from at least three animals at each experimental time.

The first variable considered was the AP activity in spleen cells from mice vaccinated with different doses of PIV. Mice immunized with 2 µg of PIV 30 days earlier were very efficient in AP, showing also a relationship between the ability of the donor cells in AP (the induction of the secondary anti-FMDV immune response in the pre-sensitized recipients) and the anti-virus immune response showed by the donors (Fig. 3).

As mentioned earlier, the characteristics of the antibody response elicited by the inactivated virus are dependent on different variables: the mass of antigen administered, the time after immunization or the immunological adjuvants used. Therefore, the presence of APC was analysed in the spleen of mice which had been immunized in different ways and had different anti-FMDV-Ab titres.
anti-FMDV-Ab response with a maximum titre at 15 days post-immunization, which almost disappeared by 30 days post-immunization (Berinstein et al., 1993; Lopez et al., 1990). Coincidental with the status of the immune response in the donors, spleen cells from mice immunized 15 days earlier were very efficient in AP, whereas no activity was observed with cells obtained at 30 days post-vaccination (Fig. 4b).

Finally, the AP activity in mice immunized with PIV mixed with different adjuvants was studied. Again, in all the considered cases, there was a close correlation between the ability of the donor cells in AP and the anti-FMDV-Ab titres in the donor mice at the time of the cell collection (Fig. 4c). This correlation suggests that the longevity and quality of the anti-FMDV-Ab response present in the host, sensitized with very different regimens of immunization, are dependent on the presence of FMDV/APC.

**Blocking of AP activity**

To understand the mechanism governing the induction of the immune response in the cell-recipient animals, the interaction between donor splenocytes and the host cells was analysed by blocking relevant cell-surface molecules. Ovalbumin, a well-studied antigen, was used as an internal control. Firstly, blocking the induction of the SA response was efficiently achieved by culturing pre-sensitized splenocytes in vitro in the presence of either an antigen-specific antiserum, an anti-MHC class II MAb or an anti-L3T4 MAb. On the other hand, MAbs reacting with pan T or pan B lymphocyte markers (Thy 1.2 and B-220, respectively), used as controls, were unable to inhibit this phenomenon (Fig. 5a). Secondly, the in vitro pre-treatment of irradiated spleen cells obtained from infected animals with the anti-MHC class II antigen MAb completely abolished the APC activity in pre-immunized recipient mice, whereas a non-relevant MAb (anti-rotavirus) did not affect the AP (Fig. 5b). Additionally, AP assays were run using splenocytes from nu/nu (euthymic) and nu/nu (athymic) mice as APC donors. The presence of APC activity was easily shown in pre-sensitized nu/nu recipients receiving APC from either nu/+ or nu/nu mice, whereas AP was not detected using athymic nu/nu mice as recipients (Table 2), indicating a need for the presence of T cells for the development of the secondary response in the recipient mouse.
environment. The results presented in Fig. 5 and Table 2 confirm that in this system, the induction of antigen-specific antibodies are strictly dependent on the phenomenon of AP which is known to be MHC-class II restricted and dependent on the T cell function.

**Table 2. Development of the antigen presentation assay in Nude mice**

<table>
<thead>
<tr>
<th>Donor*</th>
<th>Anti-FMDV titre‡</th>
<th>SD</th>
<th>Pre-sensitized receptor</th>
<th>Anti-FMDV titre‡</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nu/</td>
<td>3.5</td>
<td>0.35</td>
<td>Nu/</td>
<td>2.40</td>
<td>0.00</td>
</tr>
<tr>
<td>Nu/</td>
<td>3.4</td>
<td>0.40</td>
<td>Nu/Nu</td>
<td>0.75</td>
<td>0.21</td>
</tr>
<tr>
<td>Nu/Nu</td>
<td>3.0</td>
<td>0.45</td>
<td>Nu/+</td>
<td>2.40</td>
<td>0.00</td>
</tr>
</tbody>
</table>

(*) and (‡) are as described in Table 1.

**Discussion**

The induction of an effective long-lasting immune response in natural hosts against FMDV is a difficult task when inactivated virus is used as immunogen, even when utilizing immunomodulators in the formulation of the vaccines (Brown, 1992; Cunliffe, 1964; Morgan et al., 1980). On the other hand, immunity elicited by infection usually confers protection against the homologous virus that can last for life although, in most cases, the presence of infectious virus is only evident for a few days after infection (Borca et al., 1986; Cunliffe, 1964; Fernandez et al., 1986, Lopez et al., 1990; Morgan et al., 1980). Interestingly, the immunological requirements for the maintenance of the immune response still remain largely unknown.

Even in well-defined systems the immune mechanisms that allow an individual to produce a long-lasting antibody response following a single immunization with an antigen are not completely understood. It has been proposed that continued antibody production is probably due to repeated stimulation of antigen-specific B cells, which would require the presence of antigen for their maintenance and continuous differentiation into plasma cells (Askonas & Willamson, 1972; Feldbush, 1973; Gray & Skarval, 1988). This particular issue is still controversial and the use of adoptive cell-transfer experiments raises contradictory evidence. Thus, whereas some reports indicate that in the absence of antigen, memory B cell populations disappear from the adoptive host within a few weeks (Askonas & Willamson, 1972; Feldbush, 1973; Gray & Skarval, 1988), others demonstrate that those B cells can persist in the new host for several months after transfer without evidence of the presence of antigen (Schittek & Rajewsky, 1990; Sprent et al., 1991; Udhayakumar et al., 1988).

The immune response elicited by FMDV in mice is a good example of a long-lasting immune response induced after a very short infection. Experimental infection in mice lasts only 3–4 days, and after clearance of the viraemia no infectious virus can be found in any organ tested (Fernandez & Rajewsky, 1990; Sprent et al., 1991; Udhayakumar et al., 1988).

The presence of the life-long induced anti-FMDV-Ab response, in the apparent absence of the virus antigens, made this system an ideal model for studying the establishment of persistent antibody response. The transfer of the immune reaction from infected mice to negative recipients by adoptively transferring...
splenocytes drastically narrowed the cell populations to be analyzed in the infected mice (Fig. 1). The fact that neither infectious virus nor viral RNA could be detected in the transfer cells clearly ruled out the possibility of a persistent virus infection as a cause of the long-lasting immune response. There are several facts that lead to the idea that plasma cells are not involved in the induction of this phenomenon: (1) the appearance of anti-FMDV-Ab in the recipient always takes at least 15 days; (2) the transferred immune response (Fig. 1) clearly outlasts the reported half-life of the plasmocytes (Vidard et al., 1992); (3) the phenomenon is not radio-resistant (Table 1) whereas the function of the plasma cell is (Dobranova, et al., 1983).

Thus, the anti-FMDV response in the recipient mice must be mediated by either the donor B cells and/or by FMDV/APC, which in turn will induce the appearance of newly synthesized anti-FMDV-Ab from the host cells and/or from the co-transferred B cells. The fact that the donor cells, after being immunosuppressed through irradiation (which completely abrogates their ability to produce antibodies) (Table 1; Groups 2, 4 and 6), were still able to induce a strong anamnestic response in the FMDV-pre-sensitized recipients (Table 1, Group 7), clearly demonstrates the existence of FMDV/APC among those cells. Importantly, these FMDV/APC could be demonstrated in the spleens of naive recipients receiving the cells from the infected donors, although these splenocytes were not able to mount a new response when transferred to a second generation of naive recipients (Fig. 2). This indicates that the phenomenon is induced by a limited number of FMDV/APC originating in the infected donor. Importantly, the blocking of the induction of the antibody reaction, both in vivo and in vitro, by either anti-class II MHC antigens or antigen-specific antiseraum, as well as the requirement for T cells for the induction of the phenomenon, demonstrated that the process that leads to the immune response in the recipients is heavily dependent on the mechanism of specific AP (Fig. 5).

We have developed an adoptive cell-transfer system that allows the detection of AP in FMDV immune hosts. Employing this methodology, we have demonstrated the presence of FMDV/APC in animals that have either been infected with FMDV (Fig. 3) or immunized with different doses of PIV, with or without the utilization of adjuvants (Fig. 4). In all cases, the existence of an active anti-FMDV-Ab response closely correlated with the presence of APC among the splenocytes of the mice.

The fact that the presence of immunological response to FMDV antigens, regardless of the method for host sensitization, is invariably associated with the detection of FMDV/APC activity, strongly suggests that the presence of APC is the mechanism that mediates the maintenance of the immune responses to FMDV. This would imply that the well-documented differences in the anti-FMDV responses between animals which were infected or immunized with PIV (under very different conditions) are perhaps entirely due to the ability of each of the immunization methods to induce the appearance and persistence of FMDV/APC in the immunized host. These results could be of importance in the design and development of new vaccines. For instance, it may be more desirable to focus on improvement of the induction and maintenance of more efficient FMDV/APC rather than direct stimulation of B and T cell subsets in the host.

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References


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