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T Cell-dependent Induction of Antibody against Foot-and-Mouth Disease Virus in a Mouse Model

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SUMMARY

Nude and normal BALB/c mice were primed by intravenous inoculation of purified, infectious foot-and-mouth disease virus (FMDV) type A24, strain Cruzeiro. Frequency estimation of antigen-specific antibody-secreting cells (ASC) and Thy 1+ T cells in the spleens of immunized mice identified that the IgM response was similar for both nude and normal mice, whereas substantial numbers of both IgG ASC and Thy 1+ cells were present in normal mice only. In contrast, nude and normal mouse sera both contained IgG although the nude mouse serum was deficient in IgG1. Antigen-specific antibody could not be induced in spleen cell cultures from C57BL/6 mice after depletion of T cells with monoclonal antibody plus complement. However, the antibody response could be reconstituted if either a source of exogenous lymphokines or T cells from primed but not unprimed mice were added. Similarly, polyclonal stimulation or unprimed T cells could restore the in vitro response and thus complemented the finding of a low frequency of helper T cells in unprimed mice. Taken together, these data identify that the induction of IgG in FMDV-immunized mice is T cell-dependent and regulated by lymphokines. Furthermore, in nude mice a site other than the spleen must be responsible for the synthesis of the observed serum IgG.

INTRODUCTION

Foot-and-mouth disease virus (FMDV) belongs to the family Picornaviridae and is the cause of a highly contagious and economically important disease of domestic livestock. Protection against infection is serotype-specific and is generally considered to correlate directly with the level of neutralizing antibody in serum (Mackowiak et al., 1962; Pay & Hingley, 1987). Inactivated FMDV vaccines induce high levels of neutralizing antibody, although the immunity induced is both qualitatively poorer and the duration considerably shorter than that which follows infection. One explanation for this may be that vaccine virus is less competent with respect to the induction of a T cell response and the induction of T cell memory. In this respect, few studies have addressed the cellular basis of immunity to FMDV or the influence of infection on the regulation of the immune response.

Recently, Borca et al. (1986) reported that the adoptive transfer of primed B cells but not T cells can eliminate experimentally induced infection in mice. Whereas these workers concluded that the protective immune response against FMDV is T cell-independent, other studies (Wardley et al., 1979; Knudsen et al., 1979; Sharma et al., 1985) have demonstrated FMDV-specific proliferation of blood leukocytes, immediate and delayed hypersensitivity and the production of macrophage inhibition factor. Thus, the latter authors have implicated the induction of T cell activity as a feature of the response against FMDV. Furthermore, several picornaviruses have been shown to display T cell-dependent characteristics (Burns et al., 1975; UytdeHaag et al., 1985).

In our laboratory we are interested in the improvement of virus vaccines and, in particular, the use of synthetic peptides as vaccine antigens (DiMarchi et al., 1986). The extensive literature
on B cell regulation by T cells (e.g. Ballieux et al., 1979; Kishimoto, 1985; Melchers & Andersson, 1986), together with the perceived advantages of combining native B and T cell epitopes into a peptide antigen (Good et al., 1987), led us to question the specific requirement for T cells in the response to FMDV.

In this paper we report that the induction of FMDV-specific antibody in mice is T cell-dependent and regulated via soluble mediators. However, differences between in vivo and in vitro responses in mice suggest that both T cell-dependent and -independent pathways are induced.

METHODS

FMDV. FMDV type A24, strain Cruzeiro, was grown on monolayers of BHK-21 cells, then concentrated and purified on sucrose density gradients as previously described (Collen et al., 1984). Virus infectivity was determined by serial dilution of samples on BHK-21 cells and the virus titre was calculated as a 50% endpoint dilution by the method of Kärber (1931). Virus titres are expressed as either TCID50/ml or TCID50/organ in the case of pancreatic samples.

Mice and immunizations. C57BL/6, normal BALB/c and nude BALB/c (BALB/c-nu/nu/Ola) mice were purchased from Olac 1976 Limited. Batches of C57BL/6 mice were immunized and boosted with 10 μg purified, infectious FMDV (400 μg/ml, 10^2 TCID50/ml) as previously described (Collen et al., 1984). To study the development of virus-specific B cells, 6-week-old normal and nude BALB/c mice were immunized in triplicate by a single intravenous administration of 10 μg purified, infectious virus in 50 μl of saline into a tail vein at different times prior to assay 12 days after inoculation of the first group. The blood, serum and pancreas were monitored for the presence of infectious virus. Pancreatic tissue was disrupted in 1 ml of double strength Eagle’s MEM to avoid substantial pH changes.

Cell preparations. Lymphoid organs were removed aseptically and single cell suspensions were prepared. Erythrocytes were lysed by treatment of the spleen cells (SC) with ice-cold aqueous 0.83% (v/v) NH4Cl for 5 min on ice. Lymph node cells and SC were centrifuged through foetal calf serum (FCS), washed three times with cold RPMI 1640, suspended in the same, and held on ice until required.

Helper T (Th) cells. In some experiments, SC were treated with mitomycin C (Sigma) (25 μg/ml, 1 x 10^7 cells/ml, 20 min at 37°C), centrifuged through FCS and washed thoroughly with RPMI 1640. Cells were then used without further purification as a source of Th cells.

T cell depletion. T cells were removed by two cycles of antibody plus component in a two step depletion. The antibodies used were MAS 108c anti-Thy 1 (clone YTS 154.7) for depletion of all T cells, MAS 109c anti-Lyt 1 (clone YTS 121.5), MAS 111c anti-Lyt 2 (clone YTS 169.4) and MAS 110c anti-L3T4 (clone YTS 191.5) for depletion of specific T cell subsets. The complement used was Low-Tox M rabbit complement (CL-3051) and all reagents were purchased from Sera-Lab. Viable cells were collected by centrifugation through FCS and washed three times with RPMI 1640.

B cells. B cells were prepared as described by Möller et al. (1986). Briefly, SC were depleted of plastic-adherent cells and incubated for 20 min with rabbit anti-mouse immunoglobulin (Sera-Lab). Cells were then washed twice with phosphate-buffered saline (PBS) and incubated on swine anti-rabbit immunoglobulin (Dakopatts)-coated Petri dishes for 70 min; the untrapped cells were then decanted. The dishes were thoroughly washed with PBS containing 1% (v/v) FCS before recovery of the bound cells by treatment for 10 min with lidocaine (Sigma) (4 mg/ml in calcium–magnesium-free PBS pH 7.6) followed by vigorous pipetting. The recovered B cells were washed three times with RPMI 1640 and held on ice until required. All incubation steps were performed at 4°C.

Cell culture. The culture medium was RPMI 1640 supplemented with 5% (v/v) FCS, 10 mM-HEPES, 2 mM-glutamine, 1 mM-sodium pyruvate, 0.05 mM-2-mercaptoethanol, 100 international units/ml penicillin and 100 μg/ml streptomycin, as previously described (Collen et al., 1984). Unless otherwise stated, cells were cultured at 2 x 10^6 cells/ml using volumes of 1 ml in 24-well cluster plates or 0.25 ml in 96-well, flat-bottomed plates.

Conditioned media. Conditioned media were prepared either by culture of rat splenocytes (4 x 10^6 cells/ml) with concanavalin A type IV-S (Sigma) at 5 μg/ml for 40 h (Con A–CM) or by co-culture of equal numbers of thymocytes from 4-week-old BALB/c and C57BL/6 mice at a final cell density of 4 x 10^6 cells/ml for 40 h (T–CM). The supernatants were collected by centrifugation at 1000 g for 10 min, filter-sterilized and frozen at -20°C. The Con A–CM was supplemented with 0.1 mM-α-methyl-D-mannoside prior to filtration.

Limiting dilution assays for Th cell precursors. T cell-containing lymphocyte suspensions were cultured at different cell densities (0.0625 x 10^6 to 2 x 10^6 cells) with 3 x 10^5 T cell-depleted SC from FMDV-primed mice. The number of cells per unit volume was kept constant by the addition of mitomycin C-treated, T cell-depleted splenocytes from unprimed animals. Cells were cultured in 24-well, flat bottom plates for 6 days in the presence or absence of virus, with 48 replicate cultures per treatment. Each well was sampled individually and assayed for virus-specific antibody. The results were analysed by the method of Taswell (1981).
**ELISA.** Culture supernatants were assayed for virus-specific antibody by an indirect ELISA as previously described (Collen et al., 1984), with the modification that the diluent (PBS) contained 2% (w/v) dried milk powder (Marvel; Cadbury) (PBS–Marvel) and 0.05% (v/v) Tween 20. The isotype and subclass of virus-specific antibody was determined using a Clonotype Kit III (Sera-Lab).

**ELISA-SPOT technique.** B cells secreting FMDV-specific IgM or IgG antibodies were counted by the ELISA-SPOT technique (Sedgewick & Holt, 1983). Plates were coated as for the ELISA. After washing three times with PBS, residual binding sites were blocked by incubation with 100 μl/well of PBS containing 1% (w/v) bovine serum albumin for 45 min at 37°C. Doubling dilutions of SC starting at 10^6 cells/ml were prepared in PBS–Marvel, and 100 μl of each cell suspension was added to quadruplicate wells. Plates were incubated for 3 h at 37°C in a vibration-free atmosphere of 5% CO2 in air, after which they were washed thoroughly with PBS containing 0.05% (v/v) Tween 20. Bound antibodies were detected with rabbit anti-IgM or anti-IgG and labelled with an alkaline phosphatase–swine anti-rabbit immunoglobulin conjugate (Sigma). Spots were developed by the addition of 5-bromo-4-chloro-3-indolyl phosphate in 0.6% (w/v) agar containing AMP buffer (Sigma). The plates were observed after incubation at 37°C for 4 h and then again after overnight incubation at room temperature. The resulting blue spots were counted under the microscope.

**RESULTS**

**Development of antigen-specific antibody-secreting cells (ASC) in the spleens of normal and nude BALB/c mice**

Indirect immunofluorescent labelling with anti-Thy 1.2 monoclonal antibody (MAb) identified 36% and 0.2% splenic and 41% and 0.5% inguinal lymph node cells in normal and nude mice respectively.

Normal BALB/c mice showed an initial rise in antigen-specific IgM ASC which peaked at day 3 and declined towards days 7 to 9 (Table 1). IgG ASC were detected by day 3 and rose to a peak at around days 7 to 9. Despite the lower numbers of cells detected, the IgM ASC response of nude mice was very similar to that of normal mice. In contrast, splenic IgG ASC in nude mice showed an early rise in antigen-specific cells which, unlike the normal mice, failed to be sustained. Nevertheless, both strains of mice showed an overall increase in antigen-specific IgG-ASC during the period of study (12-fold for nude mice compared to 181-fold for normal mice).

**FMDV-specific serum antibody**

Antigen-specific IgM and IgG, but little or no IgA, were detected in the sera of both normal and nude BALB/c mice. Fig. 1 shows a series of isotype titration curves, from which it can be seen that the IgM (Fig. 1a and g) response from both strains of mice was essentially the same. Similarly, the IgG2a (Fig. 1c and i), IgG2b (Fig. 1d and j) and IgG3 (Fig. 1e and k) responses of nude and normal mice showed little difference, whereas the IgG1 response (Fig. 1b and h) was significantly lower in nude mice than in the normal BALB/c counterparts. Furthermore, IgG2b

**Table 1. ASC response of athymic and euthymic BALB/c mice**

<table>
<thead>
<tr>
<th>Time post-inoculation (days)*</th>
<th>Normal BALB/c</th>
<th>Athymic BALB/c</th>
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<tr>
<td></td>
<td>IgM</td>
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<td>0</td>
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<td>4</td>
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<td>3</td>
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<td>9</td>
<td>84</td>
<td>804</td>
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<tr>
<td>12</td>
<td>60</td>
<td>724</td>
</tr>
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</table>

* Purified A24 FMDV (10 μg) in saline was inoculated intravenously and the animals were left for the indicated time prior to assay.

† ASC determined by ELISA-SPOT technique (see Methods). The isotype specificity was assayed in a sandwich procedure and the results represent the mean of quadruplicate assays.
and IgG3 were seen to titrate out slightly earlier than IgG2a, indicating a slight bias of specific antibody towards IgG2a. A small serum IgA response (Fig. 1f and j) was detectable, in that the day 10 sera gave a higher absorbance than the day 3 sera. However, both strains of mice had a uniformly low IgA response.

**Frequency of splenic Th cell precursors**

Estimates of Th cell precursors in populations of pooled SC varied from assay to assay. Fig. 2 shows a representative analysis giving a frequency of 1 in 7.36 x 10⁵ for the primed SC population and 1 in 3.46 x 10⁷ for the unprimed population. Th cell precursor estimates for primed SC varied from 1 in 5.18 x 10⁵ to 1 in 1.06 x 10⁶ cells. Similar experiments performed using nylon wool-enriched T cell cultures provided results which were within the range defined for the whole spleen population after correction for the percentage of T cells present in the organ (results not shown).

**Activity of Th cells in vitro**

Co-culture of lymphocytes with FMDV alone did not induce a specific antibody response from T and B cells obtained from unprimed animals (Fig. 3a) or primed T cells and unprimed B cells (Fig. 3b). Equally, co-culture of primed B cells with unprimed T cells (Fig. 3c) also failed to produce antibody, although the background level was higher overall and was probably due to mitogenic activity in the batch of FCS used. Only cells from primed animals (Fig. 3d) could be induced to secrete significant levels of specific antibody. The cycloheximide control cultures indicated that the production of specific antibody was due to de novo protein synthesis.

The addition of both FMDV and Con A to unprimed B cell cultures (Fig. 3a and b) had no detectable effect. In contrast, the same treatment of primed B cells (Fig. 3c and d) with either primed or unprimed T cells resulted in similar patterns of antibody induction. Con A alone did not induce detectable antibody (results not shown). These results indicate that the in vivo priming of T cells required for the in vitro induction of antibody by FMDV alone can be replaced by non-specific activation of T cells.
T cell dependence of FMDV

Fig. 2. Helper T cell precursor frequency. Primed (○) or unprimed (●) T cells (mitomycin-inactivated SC; see Methods) were co-cultured with 0.3 × 10⁶ primed B cells for 6 days in the presence or absence of 100 μg/ml FMDV at various T cell concentrations. Individual cultures (n = 48) were assayed by ELISA and the results analysed by the method of minimum χ-squared (Taswell, 1981) using a BASIC program written for an Apple IIE microcomputer.

Fig. 3. Stimulation of 'help' for in vitro induction of specific antibody T cells (2 × 10⁶ mitomycin-inactivated SC; see Methods) were co-cultured with 0.5 × 10⁶ B cells for 6 days in the presence of medium alone (●), 100 ng/ml FMDV (○), 100 ng/ml FMDV plus 500 ng/ml Con A (△) or 100 ng/ml FMDV plus 100 μg/ml cycloheximide (---) in volumes of 1 ml. Culture fluids were assayed for specific antibody by ELISA. (a) Unprimed T cells + unprimed B cells, (b) primed T cells + unprimed B cells, (c) unprimed T cells + primed B cells, (d) primed T cells + primed B cells. Results are expressed as the mean absorbance at 492 nm of replicate assays (n = 4).

Effect of T cell depletion on in vitro antibody induction

The contribution of different T cell subsets to the in vitro induction of antibody was examined by depletion of T cells with the appropriate MAb plus complement (Table 2). Depletion of cells bearing the Thy 1.2 alloantigen or Lyt 1.2+ or L3T4+ antigens prevented the in vitro induction of antibody. In contrast, the depletion of Lyt 2.2+ cells resulted in a slightly reduced response.

T cell-replacing factor activity of conditioned media

In order to show that the activity ascribed to T cells was mediated through lymphokines, two sources of conditioned media, Con A-CM (Fig. 4a) and T-CM (Fig. 4b), were titrated on T cell-depleted SC cultures. Both conditioned medium sources showed titratable T cell-replacing
activity, although their kinetics differed. Furthermore, while no Thy 1.2+ or L3T4+ cells could be detected by immunofluorescence in T cell-depleted cultures, the titration of Con A–CM in such cultures suggested that MAb plus complement depletion may not have removed all T cells. This was supported by two observations: first, titration of Con A–CM into B cell cultures rather than T cell-depleted cultures showed no antibody induction in the absence of antigenic stimulation, and secondly, microscopical observation of cultures identified cell clusters in the T cell-depleted cultures which were characteristically distinct from the blast-forming clusters observed in the B cell cultures.

Clearance of FMDV in normal and nude BALB/c mice

The purified A24 Cruzeiro FMDV used to prime mice had an infectivity of $10^{9.2}$ TCID$_{50}$/ml (equivalent to about $10^{7.6}$ TCID$_{50}$/10 µg/mouse). Relatively little infectious virus was recovered from either normal or nude mice and no difference in the rate of virus clearance could be observed between the two strains. Furthermore, the response of individual animals was quite variable (results not shown). Infectious virus was recovered from the blood on day 1 (normal, $10^{7.74}$ TCID$_{50}$/ml, nude, $10^{7.46}$ TCID$_{50}$/ml) but not thereafter and not at all from serum. Infectious virus was isolated from the pancreas on day 1 (normal, $10^{3.23}$ TCID$_{50}$/organ; nude, $10^{3.49}$ TCID$_{50}$/organ) and day 3 (normal, $10^{3.62}$ TCID$_{50}$/organ, two mice only; nude, $10^{4.32}$ TCID$_{50}$/organ, one mouse only) but not on day 10.

![Fig. 4. T cell-replacing activity of conditioned media.](image-url)
DISCUSSION

In this study, we have used nude and normal mice, together with in vitro analyses, to examine the T cell dependence of the response to FMDV. Following inoculation of purified FMDV into nude and normal BALB/c mice we observed a splenic IgG ASC response in the latter but not the former. The nude mice contained a low but detectable number of splenic T cells at a frequency consistent with previously published data (0-2%; Raff, 1973) and, thus, it is likely that the lack of ASC class-switching in the spleens of these animals was due to the low frequency of specific T cells. In this respect, limiting dilution of antigen-specific Th cell precursors from C57BL/6 mice showed that the difference between unprimed and primed mice represented an expansion of 32- to 68-fold following immunization. Furthermore, only T cells from primed mice were able to support in vitro antibody production when used to reconstitute T cell-depleted primed B cell cultures.

In contrast to the ASC results, sera from each strain of mice contained significant amounts of FMDV-specific IgM and IgG antibodies, although in general the concentration of IgG in the nude mouse sera was lower than that in the normal mouse sera. The presence of IgG alone cannot be taken as an indication of a T cell-dependent response, since both pre-B cells and early B cells are committed to switch to IgG2b and IgG3 production (Calvert et al., 1983; Yuan & Vitetta, 1983). However, the synthesis of IgG1, IgG2a, IgE and IgA antibodies is regulated by lymphokines derived exclusively from Th cells (Snapper & Paul, 1987; Coffman et al., 1987). Thus, the presence of IgG1 (Fig. 1 b and h) and IgG2a (Fig. 1 c and i) in these sera is consistent with a T cell-dependent response against FMDV in the absence of adjuvant.

Taken together, these data support the view that FMDV is a T cell-dependent antigen. Furthermore, these observations were substantiated by the loss of an in vitro secondary antibody response after depletion of T cells from primed SC (Table 2). In vitro help for primed B cells could be provided by either specifically stimulated, primed or polyclonally stimulated, unprimed T cells. In both cases antibody was induced only in the presence of FMDV (Fig. 3). Mitogen stimulation of primed or unprimed T cells induced almost identical and near maximal responses when co-cultured with primed B cells. Although Con A can directly activate B cells (Hawrylowicz & Klaus, 1984), this interaction results in proliferation rather than antibody synthesis (Möller et al., 1986). Therefore, we believe that B cells were activated by FMDV and induced to antibody synthesis under the influence of Con A- or FMDV-induced lymphokines. Such a mechanism is consistent with the T cell-replacing activity observed with T cell–CM (Fig. 4).

Depletion of T cell subsets identified the in vitro helper phenotype as Thy 1.2+ Lyt 1.2+ L3T4+. The reduced level of antibody observed after depletion of Lyt 2.2+ cells was most likely due to direct inactivation of B cells by complement. However, it remains possible that Lyt 2.2+ T cells might contribute to the overall level of lymphokines through involvement in an autologous mixed lymphocyte response.

Contrasts between the data derived in vivo and in vitro raise several points of interest relevant to the regulatory mechanisms involved in this model. First, the presence of serum IgG but not splenic IgG ASC in nude mice indicates the importance of sites other than the spleen for serum IgG synthesis. Indeed, the frequency of T cells was higher in the inguinal lymph nodes than in the spleen (0-5% compared to 0-2%) and it has been shown that nude mice have functionally active Th cells resident in the peritoneum (Ishikawa & Saito, 1980). Second, since IgG2a has been shown to be dominant over IgG1 during virus infection (Coutelier et al., 1987), the lack of IgG1 in our nude mice together with the lack of isotype dominance in the normal mice could reflect either a more vigorous infection in nude animals or a functional defect in the T cell repertoire of nude mice. In order to assess the impact of infection the blood and pancreas were examined for the presence of infectious virus, the pancreas having been reported as the site of primary FMDV replication in mice (Platt, 1959). The level of infectious virus recovered from both organs was low compared to the input dose. However, virus did not persist in the blood beyond day 1, which was consistent with the clearance reported by Borca et al. (1986). Infectious virus was found in the pancreas of some animals on days 1 and 3 but not by day 10 and probably represented a transient or subclinical infection. However, no difference could be observed...
between nude and normal BALB/c mice in either their ability to clear virus or the establishment of virus infection. It seems unlikely, therefore, that establishment of infection was responsible for the lack of IgG1 in nude mice.

IgG1 is regulated by IL-4, a product of the Th-2 cell subset (Mosmann & Coffman, 1987) and IgG2a is regulated by γ interferon (IFN-γ) (Snapper & Paul, 1987), a product of the Th-1 cell subset. These lymphokines have a reciprocal action and an excess of either leads to down-regulation of isotypes other than that positively regulated by the lymphokine in excess. Thus, since the level of IgG isotypes other than IgG1 seems to be similar in nude and normal mice, the lack of IgG1 is probably due to a specific defect in the Th-2 subset of nude mouse Th cells. Furthermore, the lack of isotype dominance in both strains of mouse was probably due to competition between IL-4 and IFN-γ as a result of the poorly established infection.

Our experiments did not set out to address the question of protection since in our opinion infection of mice is not relevant to cattle or other natural hosts. However, we have shown here that the antibody response against FMDV is T cell-dependent and regulated by cytokines. The failure to establish a fully productive infection in these mice was probably responsible for the lack of a clear isotype dominance. Moreover, the clearance of virus at around the time when IgM is in abundance suggests that T cell-independent mechanisms may be sufficient for clearance and, thus, is in agreement with Borca et al. (1986). Nevertheless, while the initial T cell-independent response may be sufficient to clear virus we would also suggest that this is followed by a T cell-dependent phase which would be expected to influence the efficacy of the secondary response and the development and reactivation of immune memory.

How the responses described in this paper relate to those in a natural host remains to be determined. However, it is likely that both the T cell activity of a synthetic peptide vaccine antigen and the choice of adjuvant will need to be evaluated for the development of a successful peptide vaccine.

REFERENCES


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