LOCALISATION OF FOOT-AND-MOUTH DISEASE VIRUS AFTER ACUTE INFECTION IN CATTLE; A NOVEL, IMMUNOLOGICALLY SIGNIFICANT SITE.

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Control of foot-and-mouth disease involves both vaccination and controversial “stamping out” policies. Two fundamental problems remain to be understood before more effective control measures can be put in place. These problems are the foot-and-mouth disease virus [FMDV] “carrier state” and the short term duration of protection and serum neutralising antibody titres after vaccination which contrasts with the prolonged duration of immunity after natural infection. We have shown by laser capture microdissection in combination with quantitative real-time reverse transcription polymerase chain reaction, immunohistochemical analysis and corroborate by in situ hybridization that FMDV locates rapidly to, and is maintained in, the light zone of germinal centres following primary infection of naïve cattle. We propose that maintenance of non-replicating FMDV in these sites represents a source of persisting infectious virus and also contributes to the generation of long-lasting antibody responses against neutralising epitopes of the virus.

1. INTRODUCTION

A strong B-cell response, characterised by high-affinity circulating neutralising antibodies, is a crucial component of the immune response against foot-and-mouth disease virus [FMDV]. Virus is cleared rapidly from blood during the acute stage of foot-and-mouth disease [FMD], coinciding closely with the emergence of an antiviral antibody response. Viral RNA is detected in the blood of infected cattle, using real-time reverse transcription polymerase chain reaction [rRT-PCR], but becomes undetectable from as early as 3 to 5 days after onset of clinical signs. This is in contrast to pharyngeal tissue including the soft palate, nasopharynx, oropharynx, palatine tonsil and mandibular lymph node which have been shown to contain viral RNA for up to 72 days after infection (Zhang and Alexandersen, 2004). The significance of continued detection of viral RNA has not been clear since FMDV proteins have not been detected, in previous studies in these tissues, following the resolution of vesicular lesions. Importantly, FMDV proteins have not been detected previously in lymphoid tissue in vivo at any stage of infection and viral proteins have not been detected in any tissue following resolution of vesicular lesions.

A number of different pathologically relevant proteins, organisms and their products [including retroviruses like HIV, FIV and SIV, tetanus, and prion protein] have been shown to be retained on specialised cells called follicular dendritic cells [FDCs] in lymphoid tissue (Kosco-Vilbois, 2003; McGovern and Jeffrey, 2007). FDCs are non-phagocytic, non-dividing, radio-resistant cells that express surface receptors to bind immune complexes. The ability of FDCs to trap and retain antigen and infectious virus in a stable conformational state in the form of immune complexes for months or even years within germinal centres [GCs] and their intimate association with B-cells is a crucial component of the humoral response. FDCs are important for affinity maturation and memory B-cell development either through the presentation of surface-retained antigen to B-cells or by supporting B-cell proliferation and differentiation in a non specific manner (Haberman and Shlomchik, 2003). Additionally, the slow release of antigen from the surface of FDC is thought play a role in maintaining serum titres of specific antibody and studies have shown that the levels of retained antigen can regulate serum immunoglobulin levels (Tew et al., 1980; Szakal et al., 1989; Szakal et al., 1992).

2. MATERIALS AND METHODS

2.1. Challenge
The cattle examined in this study were infected by close contact with cattle infected with FMDV isolates O/UKG/34/2001 or O1BFS1860.

2.2. Laser capture microdissection
Laser capture microdissection [LCM] was performed as described previously (Allen et al., 2004), expect that ethanol fixed frozen sections were stained with a 1% solution of toluidine blue [Fluka] for 3 minutes, washed for 30 seconds in nuclease free water, dehydrated by a graded ethanol series and allowed to air dry for 10 minutes. Three replicates of the different tissues regions, each containing six microdissected samples collected in the caps of RNase-free PCR tubes [Ambion], were collected from each tissue for RNA isolation with the RNeasy Micro Kit [Qiagen] and processed by quantitative rRT-PCR.

2.3. Quantitative rRT-PCR
Reverse transcription of isolated RNA was performed concurrently with 10-fold dilution series of FMDV and bovine 28s standard RNA [Applied Biosystems]. FMDV primer/probe sets and detection were as previously described; samples with no detectable fluorescence above threshold after 50 cycles were taken to be negative (Quan et al., 2004). 28s primer/probe sets and detection were adapted from methods previously described (Valarcher et al., 2003).

2.4. Selection of monoclonal antibodies specific for conformational, non-neutralising epitopes of the FMDV capsid
Mouse monoclonal antibodies [MAbs] IB11, FC6, AD10 and BF8 raised against 146 S FMDV type O1 antigen were selected by ELISA and virus neutralising antibody test as described in the Office International des Epizooties [OIE] Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th edition, 2004. Immunoprecipitation analysis was performed as previously described by Rouiller et al (Rouiller et al., 1998). MAbs were subsequently screened by western blotting analysis and on FMDV vesicular lesions, non-infected tissue and on infected and mock-infected cells by immunofluorescence microscopy.

2.5. Immunofluorescence confocal microscopy
Samples collected at post-mortem were snap frozen in O.C.T. compound [Tissue-Tek] and stored at – 80° C until processing. Acetone fixed frozen sections of acute tissue and tissue from 29 to 38 days post contact infection were labelled in duplicate with FMDV capsid MAbs, with consecutive sections labelled with MAb 2C2, 2C1 (De Diego et al., 1997; Brocchi et al., 1998), kindly provided by E Brocchi, and isotype control MAb TRT1, TRT3 (Cook et al., 1993) and AV29, a MAb directed against a chicken antigen provided by F Davison, IAH. Additional sections were labelled with MAb D46 [specific for dark zone FDCs], MAb CNA.42 [specific for light zone FDCs, kindly provided by G Delisol, Toulouse, CHU Purpan, Laboratoire d’anatomie et cytologie pathologiques, France] (Lefevre et al., 2007), MAb CCS1 [anti-bovine CD21] (Howard and Morrison, 1991) and control MAb AV48, a MAb directed against a chicken antigen provided by F Davison, IAH. Anti-bovine CD32 MAb CCG36 and CCG37 were kindly provided by C Howard, IAH. Goat anti-mouse Molecular Probes Alexa-Fluor-conjugated secondary MAb [Invitrogen] were used and nuclei were stained with DAPI [Sigma]. All data were collected sequentially using a Leica SP2 scanning laser confocal microscope.

2.6. In situ hybridization
An optimised In situ hybridization method for the detection of FMDV was developed using digoxigenin-labelled RNA probes based on the protocol described by Prato Murphy et al (Prato Murphy et al., 1999) and optimised for frozen sections incorporating pre-hybridization blocking steps, tyramide signal amplification and alkaline phosphatase based visualization as described by Yang et al (Yang et al., 1999). Consecutive frozen sections were simultaneously probed with; 500 nt 3D antisense and sense RNA probes specific for serotype O/UKG/34/2001 non-structural protein 3D coding sequence, IgG1 antisense RNA probe [686 nt] for the CH2, CH3 and hinge region of bovine IgG1 mRNA and a 615 nt swine vesicular disease virus probe specific for structural proteins 1C and 1D coding sequence (Prato Murphy et al., 1999).

3. RESULTS

3.1. Laser capture microdissection
GC and non-GC regions of the dorsal surface of the palatum molle [dorsal soft palates], pharyngeal tonsils (Liebler-Tenorio and Pabst, 2006), palatine tonsils, lateral retropharyngeal lymph nodes and mandibular lymph nodes obtained from four cattle 38 days post contact exposure to FMDV
serotype O were selected for LCM. FMDV genome was detected consistently by quantitative rRT-PCR within the GC samples obtained by LCM. No FMDV genome was detected in the epithelium of the dorsal soft palates and pharyngeal tonsils. No FMDV genome was detected in the crypt epithelium, glandular epithelium and interfollicular regions of the palatine tonsils or the interfollicular regions of the mandibular lymph nodes and lateral retropharyngeal lymph nodes. No FMDV genome could be detected in GC samples obtained by LCM from non-infected control animals.

3.2. In situ hybridization
An optimised detection protocol with tyramide signal amplification was compared to conventional chromagenic detection (Prato Murphy et al., 1999). Tyramide signal amplification combined with endogenous peroxidase and alkaline phosphatase quenching enhanced the specific hybridization signal and reduced background signal compared to conventional detection. The FMDV 3D antisense RNA probes, control 3D sense probes and control SVD RNA probes were validated on FMDV infected and mock infected BHK-21 cells and on tissue samples collected from non-infected and FMDV infected animals. Despite the obvious signal obtained when detecting positive strand viral RNA in infected cells, it was difficult to detect negative strand viral RNA by in situ hybridization. In situ hybridization of dorsal soft palates, mandibular lymph nodes, palatine tonsils, pharyngeal tonsils and lateral retropharyngeal lymph nodes from ten animals 14 to 38 days post contact infection supported the LCM results. FMDV 3D RNA was identified in GCs of mandibular lymph node, palatine tonsil and lateral retropharyngeal lymph node sections but not in other compartments of these tissues.

3.3. Immunofluorescence confocal microscopy
To determine whether viral RNA was associated with viral structural proteins; frozen sections from the dorsal soft palates, pharyngeal tonsils, palatine tonsils, lateral retropharyngeal lymph nodes and mandibular lymph nodes collected 29 to 38 days post contact infection were analysed using a new set of virus-specific MAb s shown to be specific for conformational, non-neutralising epitopes of the FMDV capsid. These new MAb s immunoprecipitated FMDV capsid proteins, consistent with previously published antibodies, yet did not detect FMDV proteins by western blot and were non-neutralising. Also, the MAb s readily detected virus in bovine tongue during acute infection, virus infected cells and rare infected cells in lymphoid tissue during acute infection. The ability of the MAb s specific for FMDV capsid to detect immune complexed virus on the surface of cells in culture was also confirmed.

The anti-FMDV capsid MAb s gave a diffuse punctate pattern of positive labelling which was restricted to GCs within lymphoid tissue and confined to the light zone within the GC from 29 days post infection. By contrast, the FMDV non-structural proteins 3A and 3C could not be detected in any of the tissues from animals after 28 days post contact infection. Antibodies specific for 3A and 3C readily detected infected cells in FMDV vesicles and lymph tissue during the acute phase of infection and in infected BHK-21 cells, co-localising with FMDV capsid. The diffuse punctate pattern of labelled viral capsid was shown to be localised to the light zone FDC network by co-labelling with an antibody specific for light zone FDCs. Detailed analysis of in situ hybridization and immunohistochemistry showed a consistent punctate pattern. The punctate labelling pattern observed was consistent with the distribution pattern of iccosomes on FDCs (S zakal et al., 1988). This pattern is in contrast to the diffuse cytoplasmic labelling pattern of cells observed during acute infection in vivo and in infected cells in vitro.

4. DISCUSSION
We have shown that FMDV genome, using LCM and quantitative rRT-PCR, can be detected consistently in GCs within the dorsal soft palate, pharyngeal tonsil, palatine tonsil, lateral retropharyngeal lymph node and mandibular lymph node at 38 days post contact infection. Also, FMDV genome in these tissues was restricted to the GC. These findings were confirmed with in situ hybridization studies, which revealed FMDV 3D RNA in GCs of lymphoid tissues but not in other compartments of these tissues. Using MAb s specific for conformational, non-neutralising epitopes of the FMDV capsid, we identified viral structural proteins restricted to the light zone FDC network of GCs within mandibular lymph nodes, lateral retropharyngeal lymph nodes and palatine tonsils up to 38 days post contact infection, but not in the dorsal soft palates or pharyngeal tonsils. FMDV capsid was detected in mandibular lymph node GCs of all animals examined between 29 to 38 days post contact infection \([n = 22]\), including five animals where FMDV could not be recovered by virus isolation or detected by rRT-PCR analysis of oropharyngeal scrapings collected at post-mortem 29
to 34 days post infection using probang sampling cups (Alexandersen et al., 2002). These results indicate that virus is likely to persist in all cattle to some degree following infection. Although MAbs specific for FMDV non-structural proteins 3A and 3C could detect infected cells in vitro and in vivo during the acute phase of infection, no FMDV non-structural proteins were detected in any of the tissues examined from 29 days post contact infection. The absence of detectable FMDV non-structural proteins indicates that the presence of viral RNA is not associated with active viral replication. The finding of close co-localisation of viral RNA and capsid conformational epitopes, in the absence of non-structural proteins, supports the hypothesis that FMD viral particles or immune complexes are maintained in GC light zones in a non-replicating state.

The results of these studies have important implications for understanding both the mechanism of viral persistence and the ability of FMDV infection to stimulate long-lasting antibody responses. FDCs are known to be non-endocytic cells capable of capturing and retaining antigen in the form of immune complexes for long periods of time (Mandel et al., 1980; Haberman and Shlomchik, 2003). Retention of immune complexed FMDV particles within lymphoid tissue represents a possible source of the infectious material detected by pharyngeal sampling of infected cattle either by direct harvesting of mucosal associated lymphoid tissue GCs or sampling of secondary cells, for example macrophages, dendritic cells or B-cells, able to support a low level virus replication cycle in the presence of high levels of neutralising antibodies (Mason et al., 1993; Rigden et al., 2002). FDCs are notoriously difficult cells to work with, so far we have been unable to rescue infectious or immune complexed FMDV from lymphoid tissue most likely due to technical difficulties working with the bovine system. Retention of other viruses such as HIV in a replication-competent state within the light zone of GCs has been reported and the next step will require the development and interrogation of murine model systems (Smith et al., 2001). FDC-trapped HIV has been shown to represent a significant reservoir of infectious and highly diverse HIV, demonstrating greater genetic diversity than most other tissues, providing drug-resistant and immune-escape quasispecies that contribute to virus transmission, persistence and diversification (Keele et al., 2008). Retention of intact FMDV particles on the FDC network would therefore provide an ideal mechanism of maintaining a highly cytopathic and lytic virus like FMDV extracellularly in a non-replicating, native, stable nondegraded state (Tew and Mandel, 1979; Smith et al., 2001). This reservoir could serve as a source of genetically diverse viral mutants [quasispecies] able to infect susceptible cells that come into contact with the FDC network (Vosloo et al., 1996; Domingo et al., 2002).

FMDV infection in ruminants elicits an immune response that can provide protection for several years (Cunliffe, 1964) and the level of protection correlates well with specific serum neutralising antibody titres [SNTs] (Alexandersen et al., 2003). This is in contrast to vaccination, with current FMDV vaccines prepared with inactivated virus and adjuvants, providing short term duration of SNTs and protection. Long-term maintenance of elevated, specific antibody levels in mice following acute vesicular stomatitis virus [VSV] infection has been shown to be associated with the co-localisation of antigen with specific memory B-cells within long-lived GCs (Bachmann et al., 1996). VSV is a cytopathic virus that does not persist in an infectious form in mice, thus highlighting the function of FDC trapping and retention serving as a long-term repository of immunogenic antigen for maintenance of SNTs. Hence, efficient retention within the GCs of intact viral capsids, as opposed to the constituent viral proteins, may be a requirement for sustaining antibody responses relevant for providing protection against challenge. Indeed, in a recent review of the functional significance of antigen retained on FDC, Kosco-Vilbois suggests the observation that B-cell responses are independent of FDC-associated antigen is only valid in mice that are immunised with forms of antigen that leave persistent depots (Kosco-Vilbois, 2003). Therefore, we believe that long-term antibody responses detectable after FMDV infection are maintained in part by antigen persisting on FDCs. Based on the evidence presented here we suggest the persistence of FMDV after acute infection is both a consequence of the host immune response and a requirement for the long-term maintenance of protective virus-specific antibody responses.

The data described above is a summary of a manuscript currently in press for publication in PLoS ONE 2008, with the title Foot-and-Mouth Disease Virus persists in the Light Zone of Germinal Centres.

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6. REFERENCES


