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SPECIAL ARTICLE

A review of the possible mechanisms for the persistence of foot-and-mouth disease virus

INTRODUCTION

The virus

Foot-and-mouth disease (FMD) was the first animal disease to be attributed to a virus, and the second virus to be discovered [1]. It is a positive-sense, single-stranded RNA picornavirus and the sole member of the genus *Aphthovirus*. Each infectious virus particle contains a single strand of RNA approximately 8·5 kb long. This is translated into a single polypeptide which is then cleaved into the structural and non-structural virus proteins.

The capsid is non-enveloped, icosahedral in shape and approximately 28 nm in diameter. It consists of 60 copies each of the four structural proteins 1D, 1B, 1C and 1A (VP1, 2, 3 and 4). Antigenic and immunological studies have shown that VP1, 2 and 3 have surface components while VP4 is internal. This has been confirmed by X-ray diffraction which has revealed that 3D structure of the FMD virion [2]. VP1 has been shown to be the most antigenically important of the four structural proteins. It contains the 'FMDV loop', an exposed disordered area on the virion surface unique to the *Aphthoviridae* [3] which elicits a neutralizing antibody response in the host. This region also contains the highly conserved RGD sequence involved in cell attachment [4].

Serologically, FMD viruses are classified into seven immunologically distinct serotypes: O, A, C, SAT 1, SAT 2, SAT 3 and Asia 1. Animals recovered from infection with one serotype remain fully susceptible to infection with any other.

The disease

The earliest descriptions of FMD were of an outbreak in Northern Italy in 1514 [5], and in Southern Africa in 1780 [6]. At present, serotypes O, A and C can be found in South America, Africa and Asia and rarely in Europe. SAT 1, SAT 2 and SAT 3 occur in sub-Saharan Africa, while serotype Asia 1 is restricted to Asia.

Zoo sanitary measures such as movement restrictions, slaughter of affected and in-contact animals and quarantine, and vaccination programmes involving the use of inactivated whole virus vaccines have been successfully used in FMD control and eradication programmes. The elimination of FMD from Europe resulted in a decision to cease routine vaccination within the European Union (EU) in 1990–1, relying instead on import controls and quarantine to exclude FMD. Should outbreaks occur in the future they will be controlled by 'stamping out' (total slaughter) whilst emergency vaccine from strategic FMD virus antigen banks may be used as an additional control policy. The United Kingdom has been FMD-free since 1981.

FMD is an economically devastating disease affecting up to 70 species of even-

toed ungulates, both domesticated and wild. The most common route of infection of ruminants is via the respiratory tract through inhalation of airborne virus [7, 8]. Infection via the alimentary tract is also possible, although greater doses of virus are required. For example, $10^{5\cdot8}$ ID_{50} is required to infect cattle by the oral route compared to 25 ID_{50} by the respiratory route [9, 10]. Mechanical transmission of FMD virus is also possible by contaminated fomites.

The site of primary replication after infection by the respiratory route is the pharynx [11, 14–16]. Following replication in the pharynx and associated lymph nodes, FMD virus enters the bloodstream. Viraemia in cattle lasts for 3–5 days [17], and virus then spreads throughout the organs and tissues of the body to establish sites of secondary infection. This early stage of infection prior to the onset of clinical signs is associated with high titres of virus in secretions, excretions and tissues [16]. Virus may be excreted in milk and semen for up to 4 days before clinical signs of disease become apparent [11–13]. Clinical disease may develop in 2–14 days after infection depending on virus dose, strain and site of entry. Clinically, FMD is characterized by lameness, anorexia, pyrexia, salivation, reduced milk production in lactating animals and weight loss. There is frequently secondary bacterial infection of the lesions resulting in further loss of condition. Mastitis may be a sequel. In uncomplicated cases, resolution of the infection is usually complete by 14 days after infection.

FMD virus may localize in the heart of young animals, resulting in myocarditis which is usually fatal. However, FMD virus infection only rarely causes death in older animals. Cattle and other species generally recover within a short period of time, although some damage to the pancreas and other glandular tissues has been reported. Convalescent ruminant animals may harbour the FMD virus in the pharyngeal region, despite high levels of circulating antibody. Vaccinated animals exposed to virus may become persistently infected without showing clinical signs. The persistence of FMD virus in the recovered animal is called the "carrier state".

THE CARRIER STATE

A carrier animal is one from which it is possible to recover infectious FMD virus 28 days after infection. This is generally done by isolation of infectious virus from oesophageal-pharyngeal scrapings using a probang cup [18]. Present knowledge suggests that humoral antibody circulating at protective levels restricts viral replication to the tissue of the oropharynx and virus does not persist elsewhere.

Sutmoller and Gaggero [18] isolated FMD virus from 17 out of 48 animals 3 weeks to 3 months after infection. In another group of infected animals which had been vaccinated twice within 6 months, 14 out of 28 were positive virus excreters after 4 months, and 14 out of 25 were positive after 6 months. Burrows [19] later confirmed these results, isolating FMD virus from 41 out of 54 individuals 14–196 days after infection. However, the proportion of animals which become carriers varies with the severity of the virus challenge, but not the age or sex of the animal involved [20].

The duration of the carrier state varies, and is probably host species and virus strain dependent. Virus recovery in cattle has been shown 2·5 years after infection [20], whereas in sheep and goats no isolation was shown over 9 months after infection [11, 21]. Virus isolation from pigs has not been achieved more than 1

month after infection [22]. African buffalo have been shown to excrete virus for at least 5 years [23].

There is a gradual decline in the titre and frequency of virus recovery throughout the carrier period [24]. The amounts recovered in probang samples are usually at a level below that required for transmission of FMD virus to other susceptible animals by natural routes [25]. This low level virus excretion into the oropharynx may be due to cell association of persistent virus, the presence of neutralizing antibody in secretory fluids, or an altered virulence of the virus itself [26]. These results are mainly from studies under experimental or controlled conditions. It is possible that as yet unidentified "trigger factors" may result in higher levels of excretion under field conditions.

The importance of carrier animals in the epidemiology of FMD has been a matter of debate for many years. There is field evidence available both to support and refute the importance of the role of carriers. For example, Olitsky [27] observed that in Switzerland, when recovered cattle were moved to a FMD-free farm, they infected other cattle. Carrier animals were also implicated in the spread of FMD from Brazil to Mexico in 1940 [22]. Field observations by Burgi [28] and Ramon [29] indicated that a small percentage of cattle could transmit FMD to susceptible cattle at least 5–6 months and possibly up to 1 year, following an encounter with the disease. Conversely, Sutmoller [30] reported the total failure of attempts to show the transmission of virus from carrier animals following an outbreak at the National Dairy Show in Chicago in 1914. Mohler [31] also documented evidence that such animals could not transmit the disease. The epidemiological relevance of the carrier state has proved to be very difficult to establish under experimentally controlled conditions, and viral transmission by these animals remains largely unproven. Most experimentally infected carrier animals stop excreting virus spontaneously between 4–10 months after infection, although some have continued up to 14–15 months [32, 19]. Field evidence suggests that transmission within buffalo herds readily occurs from carrier buffalo to buffalo calves [33]. There are two reports of transmission from buffalo to cattle under controlled conditions [34, 35].

Routine vaccination reduces the incidence of carrier establishment in endemic areas in the field. This is the consequence of an indirect effect, as a reduced number of cases of FMD results in a lesser challenge to susceptible animals. For example in Kenya, a vaccinated area had a carrier prevalence of 0·49%, and an unvaccinated area 3·34% [36]. Trasmission from carrier to susceptible animals is probably a rare event and will be more likely when the ratio of susceptible to carrier animals is high, for example in a non-vaccinated population. Consequently, the decision of the EU to cease routine FMD vaccination in Europe in 1990–1 has increased the importance of the imported carrier animal as a potential source of an outbreak. Thus considerable effort is now being directed towards understanding the mechanism of persistence [37].

MECHANISMS OF PERSISTENCE

Salt [37] reviewed the mechanisms for viral persistence within a host and proposed that it may be mediated by changes in the virus including the production of defective interfering (DI) particles, temperature sensitive mutants, recom-

bination, integration and the infection and alteration of function of cells of the immune system. Alternatively host cell factors such as the generation of mutant viruses or lack of enzymes needed for complete viral replication may be involved. Finally persistence could be mediated by immune mechanisms which may include antibody induced antigenic modulation in the virus, immune selection, blocking factors and the generation of interferon.

IN VITRO PERSISTENT INFECTIONS

Viral mechanisms

A usually lytic virus may establish and persist by the production of DI particles, temperature-sensitive mutants, or by stimulating the production by host cells of interferon. DI particle production is an important mechanism for persistence in paramyxoviruses and rhabdoviruses, and since the DI particle was defined in 1970, they have been observed in nearly every class of RNA and DNA viruses, including picornaviruses such as poliovirus which readily produces DI particles under certain conditions; however a DI FMD virus particle has not been demonstrated [38–41].

Virus released from persistently infected cells may contain temperature-sensitive mutants, and the growth of persistently infected cell cultures at lower temperatures results in an increased amount of extracellular temperature sensitive virus [42]. Temperature sensitive mutants compete with wild type virus production, although the precise mechanism by which the parental virus is displaced is as yet unknown [43].

The production of interferon is dependent on the cell strain, and limits infection without complete elimination of the virus [44]. The initiation of persistence of FMD virus has been correlated with the production of interferon in cells [45], possibly due to an alteration of the cells. This observation is supported by recent experimental evidence that the FMD virus persistently infected cell phenotype is altered significantly from the parental strain [46].

CELLULAR MECHANISMS

It has been suggested that host cell diversity may also be important for the long term survival of FMD virus. Cellular mechanisms involved in FMD virus persistence which have been postulated include impairment of virus attachment, penetration or uncoating of the virus particles or the presence of an intracellular block. However, it has been shown by *in vitro* studies that it is a specific block to FMD virus replication which appears to be important [47].

Intracellular restriction of viral development has been documented for other picornaviruses, such as encephalomyocarditis virus (EMCV) in monkey cells [48], and mengovirus in MDBK cells [49], but the specific block which may be involved in FMD virus persistence has not yet been identified. The intracellular block was shown to limit the amount of FMD virus RNA in late passage carrier cultures of cloned BHK-21 cells [47] and was specific for FMDV as no restriction occurred with superinfection of EMCV (another picornavirus). Possible mechanism(s)

which may be involved include reduced activity of the RNA synthesis machinery caused by the presence of an inhibitor or the absence of a host factor, resulting in a direct reduction of the viral RNA present, or an indirect loss of RNA due to impairment of translation or increased RNA degradation within the cells.

Viral persistence within cell cultures may be attributed to changes in the virus as previously discussed, the cell [50], or a combination of the two [51, 52]. With respect to FMD virus, it is thought that cells that are more resistant to the virus dominate the carrier culture at late passages, and that FMD virus variants which are at least partially able to overcome the block to viral replication are selected [47]. It is possible, therefore, that cellular genes from susceptible cells, perhaps modified by mutation. may be expressed to inhibit FMD virus replication [53].

Persistent cell cultures

In vitro persistent infections are readily established in cell culture with DNA and negative- and positive-strand RNA viruses, negative-strand RNA viruses being the most easily cultured. Four types of persistent infection have been identified, distinguished by the particular virus–cell relationship [54].

First, the carrier culture is defined as one in which only a small proportion of the cell population is infected; virus is lytically released, which in turn infects only a small number of cells and which can be 'cured' of virus infection by the addition of antiviral antibody.

Second, a steady-state *in vitro* infection is defined as one in which virus and cells multiply without host cell lysis; most of the cells are infected, and virus is continually released from the cells. Antiviral antibody does not 'cure' the infection in this system.

Third, there is intracytoplasmic persistence. So far this system has been shown only with lymphocytic choriomeningitis virus (LCMV). Infectivity is associated with intracytoplasmic structures which spread by cell–cell contact, and no infectious virus is detectable in the culture medium.

Fourth, a true latent infection may occur in which the viral genome and cell genome exist in a stable relationship in the cell nucleus. Herpes virus infection of cells is a classic example.

All persistently infected cell cultures may undergo a 'crisis' at intervals during the culture period [55]. This is characterized by a sudden increase in cell lysis and virus release. However, some cells do survive and can be cultured as usual, reverting to their 'normal' persistent state.

FMD virus carrier culture

Persistent infection of BHK-21 cells with FMD virus is readily achieved, and the resulting carrier culture is easily maintained [46]. One characteristic of the carrier culture is resistance to superinfection by homologous virus. This is specific for FMD virus as attempts made to superinfect cells persistently infected with one FMD virus serotype with a second were unsuccessful. However, superinfection was achieved with EMCV, vesicular stomatitis virus (VSV) and Semliki Forest virus (SFV). Also, when cells persistently infected with serotype C were 'cured' with ribavirin [46], the cells reverted to their previous susceptible form. It has also been reported that the persistently infected cells are spontaneously cured after 100

passages in culture [56]. BHK-21 cells persistently infected with FMD viru contain a small proportion of infected cells and can be cured by treatment wit immune serum (Donn, unpublished results). Therefore the system satisfies th criteria of a true carrier culture. There is no evidence that FMD virus persisten infections established *in vitro* are due to the production of DI particles [57].

Examples of persistent picornavirus infections of cells in carrier culture include: HeLa cells with coxsackie virus A9; mice lymphoma cells with coxsacki virus B3; BHK-21 cells with FMD virus; HeLa cells with rhinovirus 2 an neuroblastoma cells with poliovirus [46, 58–62]. However, steady-state infection have also been documented for some picornaviruses, hepatitis A in fibroblasts an echovirus in WISH cells [63, 64].

The establishment and persistence of infections *in vitro* is maintained by a combination of genetic variation of the virus and of the cell. The progressive selection of a highly mutated virus and cell system leads to a coevolution of cell and virus. This may result in changes in the amino acid sequence of the persistent virus, resulting in the selection of small plaque variants, temperature sensitive mutant formation and/or unstable virions due to capsid protein alteration. Thus cell heterogeneity and a FMD virus population with a capacity for high mutation rate may be essential for the establishment of persistent infections *in vitro*. This could indicate that in the field, carriers are not only reservoirs of FMD virus, but may promote antigenic drift, and are therefore an important source of viral mutants [47, 56, 57].

IN VIVO PERSISTENT INFECTION

Viral mechanisms

FMD virus persistence *in vivo* may be mediated by various mechanisms. RNA virus genomes are highly unstable and show great potential for variation [65]. However the role which antigenic variation plays in the establishment of the carrier state is unclear. Burrows [19] reported that antigenic variation occurred in FMD virus in carrier animals, and King and colleagues [66] have also documented such antigenic variation in the field. Conversely Hedger [20] commented on the stability of FMD virus genomes in naturally occurring infections. Gebauer and colleagues [67] noted many nucleotide substitutions, 59% of which resulted in amino acid changes and caused a reduction in virus reactivity with a panel of monoclonal antibodies. However, Salt [37] concluded from experimental work with carrier cattle that immune avoidance by antigenic variation had not occurred in his investigation.

Antigenic variation also occurs in the absence of immunological pressure and is probably due to random point mutations and recombination between related FMD virus genomes [68]. Recombination has been definitively shown to occur only in poliovirus and FMD virus [69], between different strains within the same serotype, and between different serotypes [68, 70–72]. Even a low rate of mutation may result in the establishment of viral persistence. For example, a single amino acid substitution in the genome of Theiler's murine encephalomyelitis virus (TMEV) was sufficient to establish a persistent infection in the mouse brain [73], and a single amino acid substitution in the polymerase of LCMV resulted in the

organ-specific selection of variants which allowed the virus to persist within the mouse [74, 75].

Domingo and colleagues [76] proposed a 'quasispecies' model whereby each FMD virus population consists of a consensus sequence, and an equilibrium distribution of variant sequences generated by point mutations, deletions, additions and recombination. The equilibrium of the quasispecies is then thought to shift to alter the consensus sequence resulting in antigenic diversification among viruses. This variation does not depend on immune pressure to induce selection [77]. Serologically identical FMD virus isolates from a single outbreak have been shown to differ in their nucleotide sequence by up to 2·2% [78], and it is thought that this may enable sequential populations of virus to evolve, escaping the host immune system.

Genome masking or 'trans-encapsidation' has been shown to occur in FMD virus, where the RNA becomes encapsidated in the coat protein of bovine enterovirus (BEV). This has been illustrated by both *in vitro* [79] and *in vivo* [80] studies. Sutmoller and colleagues [81] have postulated that this could be responsible for the low level production of FMD virus during the carrier state.

Secretory epithelial cells are located in immunologically privileged sites, as the immune system has restricted accessibility for them [82], and thus the FMD virus may 'hide' here during persistent infection. However, so far this has not been demonstrated [38].

Cardioviruses and aphthoviruses, both picornaviruses, have a non-translated poly(C)tract. In FMD virus this is located approximately 400 nucleotides downstream of the 5′ end of the genome, and its length may vary between 80–200 nucleotides. The function of this tract and the relevance of the length variation is at present unknown. Long tract FMD virus isolates are capable of producing large, medium and small plaques in culture, while short tract isolates are unable to produce large plaques, and late passage FMD virus isolates have been shown to alter their phenotype to produce small sized plaques [83]. However, serial passage has been shown to result in an increase in poly(C)tract length of 145 nucleotides [84].

Although the size of the tract has not been directly related to viral virulence, it has been suggested that the efficiency of viral replication may be affected, in that a shorter poly(C)tract may confer some replicative advantage [83]. More recently, Escarmis and colleagues [84] postulated that any deviation from the optimum poly(C)tract length, whether increase or decrease, may affect replication and contribute to an attenuated phenotype, resulting in altered biological function which could affect virulence and favour persistence [43].

Other mutations which result in an altered biological function may also be implicated in viral persistence. For example, the single amino acid mutation which occurs in the polymerase of LCMV favours persistence by causing the cell specific selection of viral variants during infection within the host [85].

Host mechanisms

A defective immune response in a host animal may enable a virus to establish a persistent infection. In FMD there is no correlation between the development of the carrier state in the individual animal with pre-existing antibody levels [22].

Vaccinated, passively immunized and naive cattle appear equally likely to become carriers following infection [19]. Field evidence indicates that the proportion of carriers is likely to be higher in herds with a low mean antibody level [20]. Matsumoto and colleagues [86] have indicated that the secretory antibody response persists for longer and at higher levels in carrier animals. This evidence suggests that far from there being a defective immune response, in the case of local immunity there is an enhanced response during persistent infection. Other host mechanisms such as virus protection in immunologically privileged sites may also be important in persistence.

PERSISTENT MIXED INFECTIONS

Reports of different viruses infecting the same host animal simultaneously are common, although it has not always been established whether this involves multiple infections of a single cell or different cells within the same tissues. Hirano [87] reported that subacute sclerosing panencephalitis virus (SSPE) and wild-type measles virus (MV) could coinfect, and that SSPE interfered with the replication of MV. SSPE is thought to be a descendant of MV, and was shown to be dominant over MV in the culture.

Hsiung [88] showed that polio-1 and ECHO-1 viruses, both enteroviruses, could infect the same cell, which could support the multiplication of both. However, there was a greatly reduced yield of the second infecting virus. Trans-encapsidation involving bovine enterovirus and FMD virus (both picornaviruses) has been described above. Clearly, for this phenomenon to occur, the viruses must exist in the same cell and replicate together.

There are 24 different serotypes of bluetongue virus (BTV), and it has been estimated that one third of all infected animals contain more than one serotype [89, 90]. Samal and colleagues [91] showed that BTV serotypes 10 and 17 coinfected the same cell, and were able to undergo genetic reassortment in the host.

Mixed infections in individual animals also occur with different serotypes of FMD virus. Experimental dual infections have been established with serotypes O and A in calves [92]; O, A and C in cattle [93] and O and A in guinea pigs [94]. SAT 1, 2 and 3 have all been isolated from carrier African buffalo [95], and Hedger [96] showed the transfer of SAT 2 from a carrier buffalo to an animal already infected with SAT 1. In all these cases, one dominant serotype was present, and the second virus was not eliminated but replicated within the host.

The occurrence of dual infections in the field involving strains of FMD virus serotype Asia 1 alongside strains of serotype O in Saudi Arabia has been recently reported [97]. Studies showed that the viruses persisted together in a stable manner within the country for several years, and that although the O serotype was always dominant, Asia 1 was never completely eliminated. The source of the Asia 1 virus, which was thought to be absent from Saudi Arabia, is unknown. Sequence analysis of the Asia 1 strains suggest that the virus is related to a vaccine strain used in this area and to an isolate from a FMD virus outbreak in Turkey in 1973. Preliminary animal experiments have shown that the two virus serotypes can be

transmitted together (Davidson and Salt, unpublished). Persistent mixed infections of sheep in the field involving FMD virus serotypes O and A have also been recently reported [98].

Intuitively, a persistent mixed infection involving two serotypes of FMD virus should not occur under natural conditions, as one should have a selective advantage over, and therefore eventually eliminate the other. However, a possible mechanism of persistence based on dual-serotype infection would be differential cell tropism, as occurs in LCMV, where during a prolonged period of persistence in mice, viral variants with a growth advantage in different cell types emerge [85]. This mechanism cannot yet be related to FMD virus persistence as the cellular site or sites of persistence within host animals has not been determined.

Another possibility is that each serotype utilizes different receptors on the surface of cells. Colonno [99] determined that all FMD virus serotypes share at least one common cellular receptor which is unrelated to those used by poliovirus and EMCV, although there are also receptor sites unique for each serotype involved. Trans-encapsidation may also be a mechanism by which two serotypes could co-exist within the same host.

CONCLUSIONS

Despite the recognized importance of carrier animals in the epidemiology of FMD, the mechanism(s) of viral persistence within the host animal has yet to be elucidated. The use of persistently infected carrier cell cultures has enabled factors such as viral antigenic variation, production of mutant viruses, intracellular blocks to viral replication and the role of interferon in the persistence of FMD to be investigated. However, possible interactions with host mechanisms cannot be modelled within this system.

The identification of the cellular site or sites of FMD virus persistence should provide vital clues and increased opportunities to investigate the mechanism(s) which are involved. It is likely that the establishment and maintenance of the FMD virus carrier state involves a combination of cellular, viral and host-animal factors.

The mechanisms for the maintenance of multiple serotype infections under natural conditions in the field may prove to be a more difficult problem to solve, as account will have to be given of competition between the two viruses.

The need to understand the mechanism(s) of establishment and maintenance of the carrier state in FMD has become more important due to the greater risk which these animals represent, particularly as routine vaccination within the European Union has ceased and the livestock population is now highly susceptible to infection.

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