0.4 Foot-and-mouth disease

0.4.1 General features[1-5]

Foot-and-mouth disease (FMD) is an acute systemic infection affecting even-toed ungulates, both domesticated and wild, including cattle, swine, sheep and goats. FMD generally involves mortality rates below 5%, but even so it is considered the most important disease of farm animals since it causes important decreases in livestock productivity and trade. The main route of infection of ruminants is the inhalation of airborne virus, but infection *via* the alimentary tract or skin lesions is also possible, although requiring higher doses of virus. After primary replication in the pharynx, the virus enters the bloodstream and, following a 3 to 5 days period of febrile viræmia, it spreads throughout the organs and tissues where new sites for secondary infection are established. Some clinical symptoms of FMD are fever, anorexia, weight loss, lameness, salivation and vesicular lesions (mouth and skin). Although FMD only rarely causes death in adult animals, the virus can cause severe lesions in the myocardium of young animals, leading in this case to high mortality rates[1-3].

An asymptomatic persistent infection can be established in ruminants for periods of a few weeks to several years as a consequence either of the acute infection or of vaccination with live-attenuated virus. Animals affected by this long-term persistent infection are known as *carrier animals* and are an important reservoir of the FMD virus in nature. Also, it has been suggested that carrier cattle are a possible source of FMD outbreaks by virus transmission to susceptible animals. The impossibility to cure carrier animals by vaccination, together with the extraordinary genetic and antigenic complexity of the FMD virus, are major drawbacks for the control of the disease[1-5].

0.4.2 Natural distribution of FMD

The earliest reports on FMD were descriptions of outbreaks in Northern Italy in 1514 and in Southern Africa in 1780, written by Fracastorri[6] and Le Vailant[7], respectively. Seven immunologically different serotypes of the FMD virus are known, namely A, O, C, Asia-1, South-African Territories (SAT) -1, -2 and -3, which comprise more than 65 subtypes. The global distribution of the disease in 1997 was as represented in Fig. 0.18, with no significant changes over the last 30 years. FMD is endemic in South America, sub-Saharan Africa, India and Middle/Far East[8]. Countries such as Chile, French Guyana, Guyana and Surinam have been FMD free for the last decade, while the members of the Mercasur (Argentina, Uruguay, Paraguay and Brazil) have greatly improved the control of the disease through vaccination programmes[8]. In sub-Saharan Africa, the control of FMD has been motivated by the exportation of beef to Europe. However, there is occasional spread of the disease from the African buffalo, which is usually restricted to game parks. On the other hand, poor surveillance and diagnostic facilities as well as deterioration of some control programmes are causative of FMD spread to domestic cattle, including North-African countries like Tunisia, Morocco and Algeria[8].

Figure 0. 18 Estimated world distribution of FMD in 1997. Dark zones represent regions where the disease is endemic, striped zones regions where the disease is controlled and under vaccination programmes and light zones are FMD free (adapted from reference 8).

The control of the disease in India and other countries in the Far East is very difficult due to the extremely large number of sheep, goats and cattle and to the poverty of many of the farmers. New outbreaks occurred in Asian countries where the disease had been controlled (Malaysia, Philippines, Japan) and FMD was recently introduced in Taiwan[8]. The uncontrollable movement of livestock between countries of the Middle East has made it impossible to effectively control the disease in this region of the world. Partial control of FMD was achieved only in Israel, upon immunisation with vaccines produced in Europe. Of concern to Europe has been the situation in Turkey, since this is the traditional route by which FMD enters the Balkans[8]. Sanitary measures such as movement restrictions and quarantine, total slaughter of affected and in-contact animals ("stamping out") and extensive vaccination employing inactivated whole-virus have been successful in the control and eradication of the disease in Europe (Fig. 0.19), which led to the decision of the European Union to cease vaccination in 1991. This decision was followed, for the sake of trading agreements, by the remaining European countries, and control of imports, quarantine and "stamping out" replaced vaccination as the measures to exclude the disease. Nevertheless, several outbreaks have been reported in Europe since 1991, namely in Bulgaria (1991, 1993, 1996), Italy (1993), Greece (1994, 1996), Russia (1995), Albania (1996), Macedonia (1996), Kosovo (1996) and the Turkish Thrace (1995, 1996)[8]. In the particular context of the Iberian Peninsula, the last recorded outbreak occurred in Spain during 1986. Although the peninsula has been FMD-free since then, the large (and insufficiently controlled) flow in persons and goods from and into Northern African territories is a cause of grave concern for the animal health authorities, even if not explicitly acknowledged.

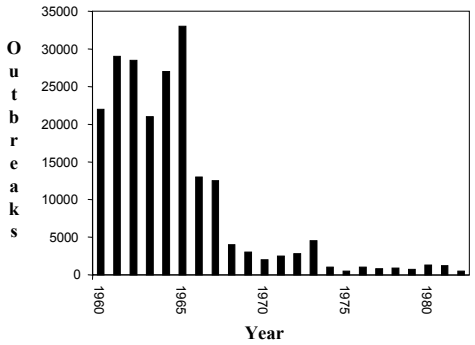

Figure 0. 19 Estimated FMD outbreaks in Europe from 1960 to 1982 (reproduced from reference 1).

0.5 Foot-and-mouth disease virus

0.5.1 The virus particle

Foot-and-mouth disease virus (FMDV) was the first recognized viral pathogen (by Loeffler and Frosch in 1898[9]) and is the sole member of the genus *Aphthovirus* belonging to the *Picornaviridæ* family. The viral particle, or virion, contains a single-stranded RNA of positive polarity, approximately 8500 nucleotides long. The RNA is covalently linked to a small protein, VPg, at its 5' terminus and translation of the RNA yields a single polypeptide (L-P1-P2-P3) which is then cleaved into the structural (from the P1 region) and non-structural (such as the viral-specific protease 3C and the viral-specific RNA polymerase 3D) proteins. The virus capsid is non-enveloped and has icosahedral symmetry with a diameter of approximately 300 Å, consisting of 60 copies of each of the structural proteins VP1, VP2, VP3 and VP4 (Fig. 0.20). While the first three structural proteins (MW≈24 kDa) have surface components, the fourth (MW≈8.5 kDa) is internal. The virion is also usually composed by one or two units of VP0, the precursor of VP2 and VP4[10,11].

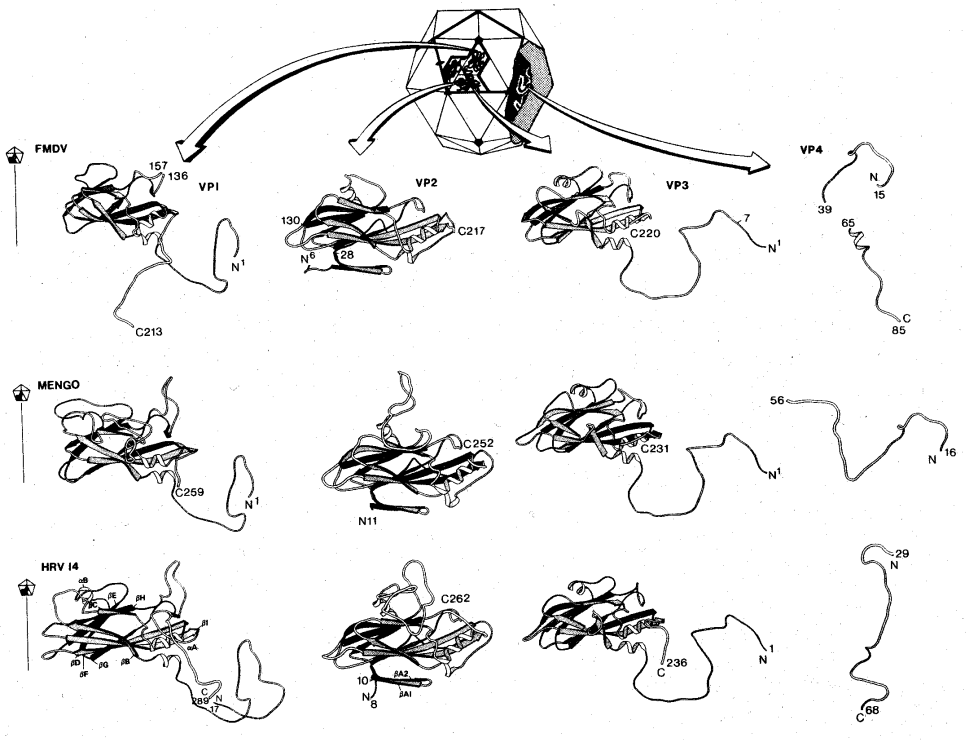

Figure 0. 20 Illustration of the structure of three picornaviruses (FMDV, Mengo and HRV14) and their capsid proteins VP1-4 (reproduced from reference 11).

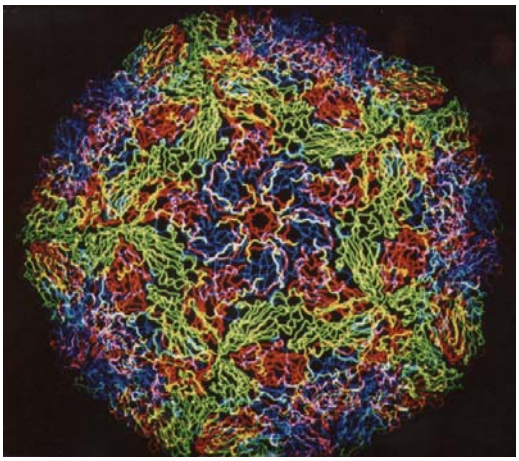

Figure 0. 21 Structure of the viral capsid for FMDV C-S8c1 (only α C are represented); VP1 – 4 are represented in blue, green, red and yellow, respectively (reproduced from reference

0.5.2 Architecture of the FMD virion

The structure of the FMDV particle was first resolved for serotype O_1 BFS 1860 by X-ray diffraction analysis[11]. Since then, other serotypes of FMDV have been crystallised and analysed, allowing some of the phenotypic (*e.g.*, high buoyant density in CsCl, acid lability) and serological (immunological and antigenic) properties of the virus to be explained from a structural point of view[12-15]. The overall shape of the outer virus surface is approximately spherical and relatively smooth. The virus has icosahedral symmetry (Fig. 0.21); each asymmetric unit (1/12 of the particle) is a pentamer[11-15] formed upon assembly of five copies of the biological protomer of FMDV[16]. The arrangement of VP1, VP2 and VP3 in the biological protomer is as represented in Fig. 0.22, where the internal VP4 is not displayed.

The viral proteins VP1 – 3 of FMDV are quite similar in size, position, orientation and tertiary structure to those of other picornaviruses, with VP1 showing the most significant rearrangements. VP4 is the most variable protein among picornaviruses, the one belonging to FMDV being the larger (Fig. 0.20).

0.5.3 Antigenic structure of FMDV

It has long been known that the main cell attachment site and the immunodominant region of FMDV are both located on a solvent exposed region at the surface of the virion, namely in trypsin-sensitive areas of VP1[17,18]. Earlier serological studies showed that different serotypes of FMDV shared a highly variable region of VP1, comprising residues 135 to 155 (Fig. 0.23)[19], as one of the major antigenic sites of the virus. Several overlapping B-cell epitopes are located within this region and are able to induce both neutralising and non-neutralising antibody responses[19-23]. The high sequence variability found in this region accounts for the low cross-reactivity observed among different serotypes[21-23].

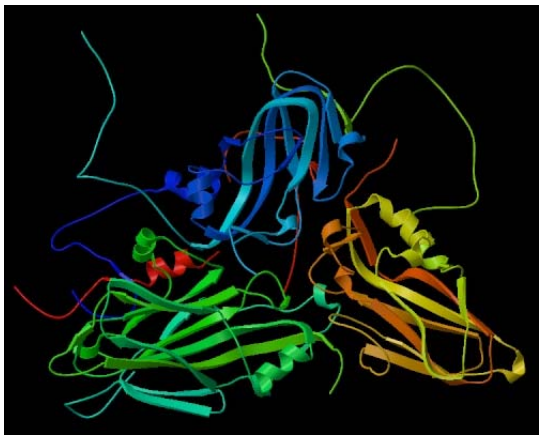

Figure 0. 22 Ribbon protein diagram of the FMDV C-S8c1 protomer composed of proteins VP1 – blue, VP2 – green and VP3 – yellow (reproduced from reference 16).

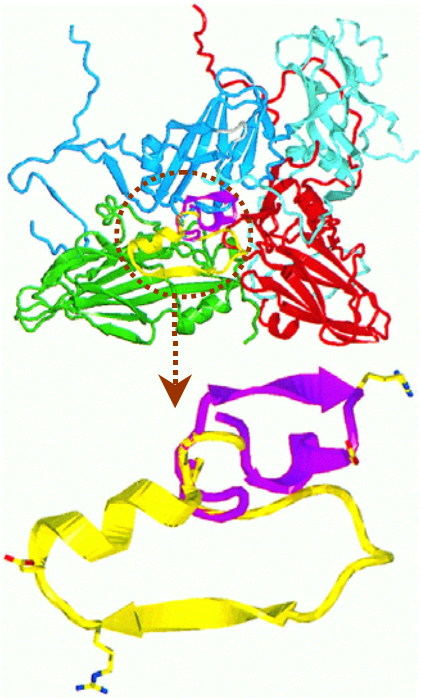

Figure 0. 23 Localisation of the GH loop within the FMDV protomer (above) and detailed illustration of the conformation of this loop (below) – yellow for isolate O_1 BFS; magenta for isolate C-S8c1. The RGD motif is shown in detail (reproduced from reference 51).

This immunodominant region was seen to correspond to the loop which connects β-sheets G and H of the VP1 β-barrel, named the *GH loop*[11-15]. Since the first evidences pointing to the relevance of the GH loop in both the infectivity and immune response in FMD, an enormous volume of research has been focused on this region[14,19-55]. Unfortunately, the first crystal structure of FMDV (strain O_1 BFS) showed this region to have very low electron density[11], indicating high mobility and thus lack of a defined structure. Based on the assumption that such disordered conformation was dependent on a native disulphide bond linking Cys134 of VP1, at the base of the loop, and Cys130 of VP2, the crystal structure of FMDV O_1 BFS was analysed under reducing conditions and the conformation of the loop was thus resolved[12] (shown in yellow in Fig. 0.23). Other important antigenic and immunogenic sites have been identified in several FMDV serotypes; for instance, the C-terminal stretch of VP1 (which, together with the GH loop, defines the main antigenic site 1 in serotype O), or sites involving different loops from the three accessible viral proteins (*e.g.* sites 2, 3 and 4 of FMDV O)[28,56-59]. The absence of cross-reactivity between the different types of FMDV, together with the lack of steric hindrance between serotype-specific mAbs in competition experiments, clearly show that antigenic sites in these serotypes are topologically independent from each other. Resolution of the crystal structures of other FMDV variants, such as FMDV C-S8c1[15], or peptide/virus – antibody complexes[33,36,42,51,52], provided further evidence of such topological differences, as shown in Fig. 0.23.

0.5.4 FMDV cell attachment sites: the Arg-Gly-Asp motif

Studies on surface topology, sequence conservation and inhibition of cell attachment of different picornaviruses have shown that the majority of these pathogens share a common strategy for hiding their cell attachment sites from the immune system. Such sites are usually placed inside canyons or pits, out of reach from antibody footprints[60]. The absence of any such canyons or pits in the smooth FMDV surface[11-15], as well as the existence of a highly conserved Arg-Gly-Asp (RGD) motif within the hypervariable GH loop of VP1[11-59], led to suspect that this motif could have a key role in infectivity, since RGD is known to promote cell attachment in several different systems[61]. Immunochemical and structural studies have shown that the RGD motif is, in fact, critically involved in FMDV infectivity, upon cell attachment *via* the integrin $\alpha_v\beta_3$, the vitronectin receptor[18,27,36,41,42,62-70]. Being placed in a highly exposed region of FMDV, the RGD motif has been surprisingly conserved

among the different serotypes, in spite of the high immune pressure exerted on this region. The strategy of FMDV to elude antibody recognition is based on surrounding RGD with hypervariable residues within a disordered loop. Thus, a mechanism for escape from antibody neutralisation would involve subtle structural modifications which preserve the integrin-recognisable open-turn conformation of the RGD triplet (Fig. 0.23)[41,42,44,62-69].

Despite its obvious relevance, the RGD motif is not the only possible route for FMDV to be internalised by the host cells[70-77]. Increasing evidence that FMDV clones lacking the RGD triplet can infect host cells has made the essentiality of this motif questionable. In fact, it is now known that there are at least three different mechanisms for cell recognition by FMDV. Apart from the RGD-integrin mechanism, there are isolates of FMDV which use heparan sulphate (HS) as the predominant cell surface ligand[72-75] (*e.g.*, certain strains of FMDV O_1, cell culture-adapted FMD viruses) and even others which can establish RGD- and HS-independent infections[76].

It has also been reported that FMDV can cause infection *via* the antibody-dependent enhancement pathway, in which FMDV bound to virus-specific antibodies could enter cells via the Fc receptor, thus bypassing the RGD mechanism[70,71].

0.5.5 Antigenic and genetic variability of FMDV

RNA viruses are characterised by an error-prone RNA replication, which gives them great potential for variation[1,10]. In FMDV genomes, the sequence homology between different serotypes can be as low as 25-40% while homologies between subtypes of a same serotype are usually above 60-70%[23]. Natural populations of FMDV from a single disease outbreak have been shown to be heterogeneous and, moreover, "individual" isolates have been reported to include two different nucleotide sequences. The high variability of FMDV (Table 0.3) led to the proposal that FMDV natural populations are *quasispecies*, *i. e.*, pools of variant genomes statistically defined but individually indeterminate[1,10,78]. High mutation rates during replication allow FMD viruses to continuously evolve and adapt to new environments. Although most mutations will be detrimental and eliminated by natural selection (negative selection), others can be of value under the particular conditions where the virus is replicating and are therefore selected (positive selection)[1,10,75,78-87]. Despite the high heterogeneity of FMDV populations, there is a potential for long-term conservation of sequences due to the continuous selection of a same consensus sequence in a situation of equilibrium[79]. Whenever this equilibrium is ruptured, rapid evolution and selection of new *master* sequences take place[1,10,79].

One of the most troubling consequences of genetic variability is antigenic diversity. Immunochemical studies have shown that isolates of the same geographical and chronological origin as well as viral clones derived from single isolates may be antigenically distinct[10,12,30,57,70,79-95]. Antigenic variants have been isolated under variable conditions, such as in partially immune animals, persistently infected cattle[4] and in cell culture[96-98], in the latter case both in the presence or the absence of immune pressure[99,100]. Therefore, antigenic variants result from the high mutation rates during RNA

replication and from the negative selection of most of the mutant phenotypes. This would mean that substitutions at antigenic sites such as the FMDV GH loop are very likely to occur, since these are disordered, flexible, and therefore, permissive sites, not subject to intensive negative selection.
This antigenic diversity has serious implications in vaccine design since synthetic vaccines should include multiple independent epitopes in order to decrease the probability of selection of FMD viruses resistant to the immune response.

Table 0.3 Variability of FMDV (reproduced from reference 12 and based on data from references therein).

Genetic heterogeneity	Substitutions/genome
During a disease outbreak	
Among consensus sequences of different isolates	***60 – 70***
Among consensus sequences of contemporary isolates	***2 – 20***
Among individual genomes of one isolate	***0.6 – 2***
Of clonal populations in cell culture	
Among consensus sequences of independently passaged plaque-purified viruses	***14 – 57***
Among individual genomes of clonal, passaged population	***2 – 8***
Frequency of mAb-resistant mutants	
In viruses from lesions of infected animals	$2\times10^{-6} – 2\times10^{-5}$
In viruses from cell culture fluid	4×10^{-5}
Evolution	Substitutions/nucleotide/year
Rate of fixation of mutations	
Acute disease	$<4\times10^{-4} – 4.5\times10^{-2}$
Persistent infection	$9\times10^{-3} – 7.4\times10^{-2}$

0.6 The development of anti-FMDV vaccines

0.6.1 Conventional vaccines

Vaccination has been one of the most powerful tools for efficient control of infectious diseases such as poliomyelitis, measles, yellow fever and smallpox, the latter having been totally eradicated world-wide. Conventional vaccines are whole-virus vaccines where attenuated variants or inactivated viruses are employed. The possibility to control viral RNA *quasispecies* with classical vaccines relies on two important factors: ***i.*** attempts of the virus to escape immune response upon mutation lead to non-viable phenotypes which cannot adapt to the environment, and ***ii.*** the constant actualisation of vaccine strains to include field variants from new outbreaks provides a broad coverage of the genetic and antigenic heterogeneity found in the field. Nevertheless, high mutational rates are still an obstacle to the efficacy of RNA viral vaccines. Also, on a more practical level, not all viruses are easily grown in cell culture, a fact that can often prevent the production of classical vaccines, such as hepatitis A[101].
Most vaccines against FMDV are prepared by growing the virus in surviving bovine tongue epithelial fragments, pig or calf kidney cell monolayers, or in baby hamster kidney cell culture and subsequent inactivation with ethyleneimine (aziridine). The inactivated virus is then adsorbed onto aluminium

hydroxide and mixed with saponin prior to inoculation; vaccine delivery can also rely on emulsions with an oil adjuvant. These classical anti-FMDV vaccines, given as a single dose, have been effective in the control of the disease in Western Europe[3,8,101-105].

The need of cold chains to keep viral vaccines at low temperatures in order to preserve their immunogenicity is one of the reasons for the unsuccessful vaccination programmes in countries with difficult terrain and climate conditions (*e.g.*, tropical countries). Other disadvantages of whole-virus vaccines arise from occasional deficient inactivation of the infective particle and consequent escape to the field, causing new FMD outbreaks and eventually establishing persistent infections in cattle, which act as important reservoirs and factories of new variants. But, clearly, the most important problem of anti-FMDV classical vaccines comes from the high antigenic diversity of this virus, since convalescent animals recovering from infection with a particular serotype are not protected against other serotypes. Furthermore, each serotype consists of a wide spectrum of variant isolates and often the virus strain used to prepare vaccines against a certain serotype does not offer the same degree of protection against other isolates of the same serotype. Moreover, adaptation of an outbreak virus to growth in cell culture can lead to the selection of variants that are antigenically different from their parental virus[1,2,10,12,30,70,79-98].

0.6.2 Synthetic vaccines

In view of the difficulties posed by conventional vaccines, the development of synthetic, molecularly engineered vaccines has become a priority for the control of viral diseases. In particular, the use of peptide-based synthetic vaccines offers significant advantages over classical procedures in terms of stability, availability, safety, purity and cost[101,106]. These benefits are not easily achieved, however. Thus, in order to design effective candidate vaccines, the antigenic and immunogenic determinants of the pathogen must be adequately understood.

Intensive research has been focused on FMDV B-cell epitopes with the hope that they could be mimicked by short linear peptides capable of eliciting protective virus-specific immune responses[20,25,26,39,43,45,107-115]. As mentioned before, early studies allowed the recognition of major antigenic sites located within protein VP1, namely the GH loop (antigenic site A) and the C-terminal region (antigenic site C). Peptide vaccines based on site A or on constructs including both A and C sites (the so-called "DiMarchi" peptides, Fig. 0.24) induced significant levels of anti-FMDV neutralising antibodies and protected either guinea-pigs or natural hosts (pigs and cattle)[116-118].

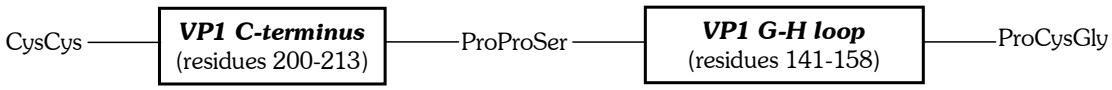

Figure 0. 24 The "DiMarchi" peptide antigen: the VP1 C-terminal and GH loop regions from FMDV O_1 Kaufbeuren were brought together in a linear construct; a ProProSer spacer was used to induce a turn and Cys residues were placed at each terminus to allow oligomerisation and bypass the use of a carrier protein.

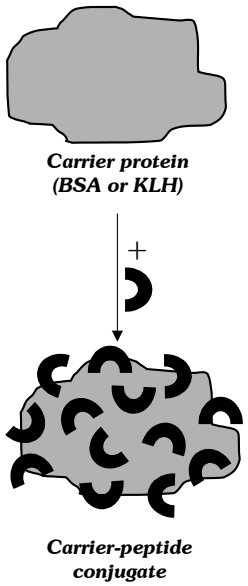

Figure 0. 25 Representation of a peptide-carrier protein conjugate (peptide represented as a black "loop").

The immunogenicity of synthetic FMDV antigens was shown to be generally lower than that of classical vaccines in natural hosts. Also differences between anti-virus and anti-peptide immune responses were detected, namely, the good correlation between neutralising activity of anti-virus sera and host protection was not always well established in peptide-immunised animals[119-123]. Attempts to increase the immunogenicity of small FMDV peptides include the design of constructs containing tandem repeats of the linear peptide, attachment of peptide to carrier proteins such as bovine serum albumin (BSA) and keyhole limpet hæmocyanin (KLH)[107-115] (Fig. 0.25) or insertion in scaffolds such as multiple antigenic peptide (MAP) systems[124] (Fig. 0.26), recombinant proteins (*e.g.*, β-galactosidase from *Escherichia coli*[35,40,125,126]) and hepatitis B virus core (HBc) protein which self-assembles into a spherical virus-like particle, the hepatitis B core antigen (HBcAg)[67,101] (Fig. 0.27). Recombinant technology is also important in protection against FMDV, as shown by recent results using recombinant viruses or transgenic plants where VP1 or the precursor polypeptide P1 of FMDV capsid proteins have been inserted[127-130].

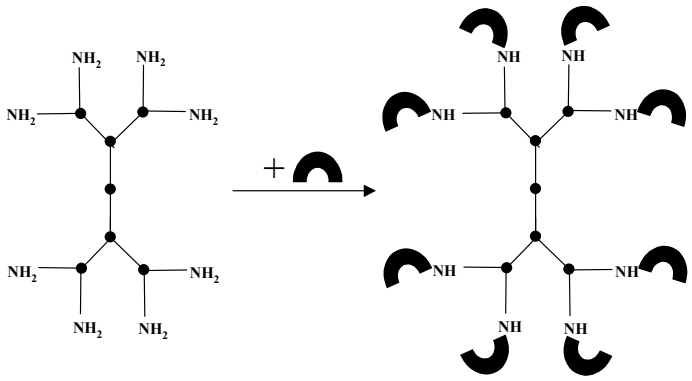

Figure 0. 26 Multiple antigenic peptide (MAP) system: a poly-lysine scaffold is used to present in a single chimera several copies (8, in the present case) of the synthetic antigen, represented as a black "loop" (adapted from reference 101).

In terms of antigenicity and immunogenicity, comparison between different peptide vaccine candidates clearly shows that peptide presentation and orientation are important[25,35,40,101,125,126,131]. Thus, insertion of a peptide reproducing site A of FMDV C-S8c1 on different solvent-exposed loops of the homotetrameric enzyme β-galactosidase yielded different antigenicity levels of the resulting chimeras, some of them more antigenic than the corresponding KLH conjugate[35,40,125,126]. These results prove the sensitivity of anti-FMDV antibody responses to peptide conformation, regardless of the localisation of antigenic site A on a linear and flexible loop. Another evidence of such dependence on orientation was reported by Schaaper *et al.*, who observed anti-peptide immune responses dependent on the peptide-carrier coupling method[131].

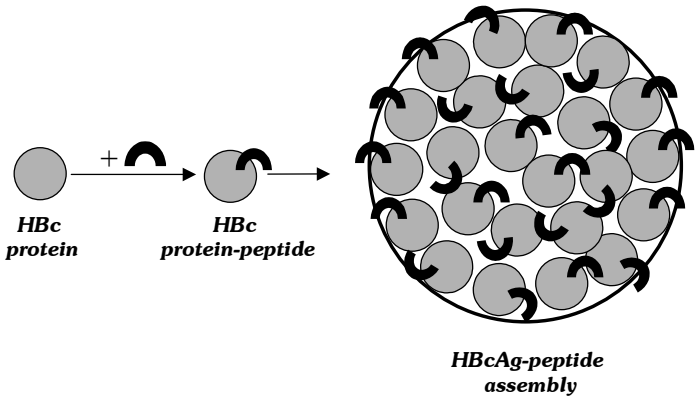

Figure 0. 27 Antigenic peptide (black "loop") insertion into hepatitis B virus core protein and self-assembly of the latter into hepatitis B core antigen (HbcAg) particle (adapted from reference 101).

Despite these differences, short linear and cyclic peptides have been shown to reproduce rather faithfully the features of antigenic site A from different isolates, including C-S strains [29,31,34,36-38,41,44,49,50]. This opened the possibility of analysing in detail the effects of amino acid replacements found in natural isolates and the repercussions of antigenic variation in the field[132,133]. Moreover, it allowed an extensive screening of the effects of single-point replacements of amino acids spanning the entire GH loop[41] (Fig. 0.28) which, together with the recently resolved structures of some peptide-antibody complexes[36,42,44], provided further insight into the mechanisms of interaction between the GH loop and anti-FMDV neutralising antibodies. The knowledge of such mechanisms at the molecular level can provide the basis for the design of FMDV peptides with strong antigenic character, suitable to be inserted in constructs including T-cell epitopes and other immunogenic components to produce efficient synthetic anti-FMDV vaccines. Recent advances with retro-inverso FMDV peptides[135-138] (increasing peptide resistance to host proteases) and with synthetic models of important discontinuous antigenic sites (site D from FMDV C-S8c1 isolate)[59] are also encouraging regarding the future of fully synthetic anti-FMDV vaccines.

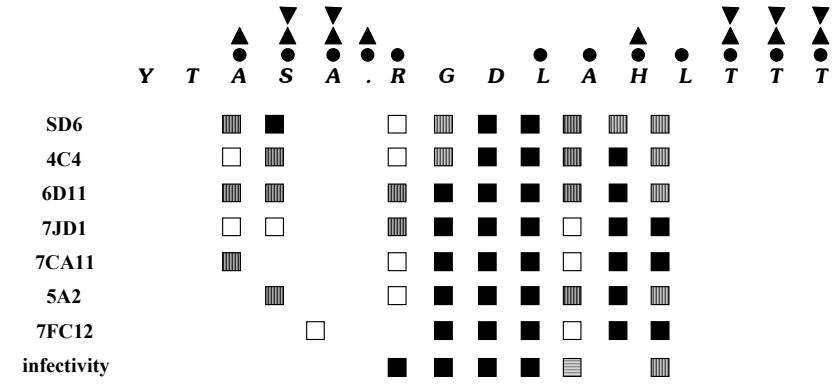

Figure 0. 28 Sequence of the GH loop from C-S8c1 clone of FMDV (adapted from reference 134).

Above the sequence: (●) variable residues found in 50 field isolates of serotype C FMDV; (▲) residues found replaced in 97 laboratory FMDV mutants (89 of them derived from C-S8c1) selected by antibodies; (▼) replaced residues found after 25 independent passages of FMDV in cell culture, in the absence of immune pressure. *Below the sequence*: Average effects of single-point replacements within site A on antigenicity towards 7 anti-FMDV monoclonal antibodies, where a black box stands for $IC_{50}>100$, a vertically striped box for $30<IC_{50}<100$ and a white box to $IC_{50}<5$. The last row represents the average effects of single-point mutations within the GH loop on inhibition of infectivity (FMDV C-S8c1), where a black box stands for $IC_{50}>300$, a vertically striped box for $30<IC_{50}<300$ and a horizontally striped box for $5<IC_{50}<30$.

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