The encephalomyocarditis virus

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The encephalomyocarditis virus (EMCV) is a small non-enveloped single-strand RNA virus, the causative agent of not only myocarditis and encephalitis, but also neurological diseases, reproductive disorders and diabetes in many mammalian species. EMCV pathogenesis appears to be viral strain- and host-specific, and a better understanding of EMCV virulence factors is increasingly required. Indeed, EMCV is often used as a model for diabetes and viral myocarditis, and is also widely used in immunology as a double-stranded RNA stimulus in the study of Toll-like as well as cytosolic receptors. However, EMCV virulence and properties have often been neglected. Moreover, EMCV is able to infect humans albeit with a low morbidity. Progress on xenografts, such as pig heart transplantation in humans, has raised safety concerns that need to be explored. In this review we will highlight the biology of EMCV and all known and potential virulence factors.

Description of EMCV

History. The encephalomyocarditis virus (EMCV) was first isolated in 1945, by Helwig and Schmidt in Miami, FL.[1] It was from a captive male gibbon that died suddenly of pulmonary edema and myocarditis. Mice inoculated intravenously, intraperitoneally, subcutaneously, intracranially or by intranasal instillation with filtered edema fluid from the gibbon developed paralysis of the posterior members and a myocarditis followed by death in a week. The pathogenic agent was at that time named the encephalomyocarditis virus.

In 1948, a Mengo virus was isolated by Dick et al.[2] in the Mengo district of Entebbe in Uganda. It was isolated from a captive rhesus monkey that had developed hind limb paralysis. In 1949, cross sero-neutralization studies showed that Mengo, EMCV were antigenically indistinct from each other, meaning they are part of the same species. However, they were distinct from Theiler's murine encephalomyelitis virus (TMEV) another cardiovirus (compare classification) that is responsible, depending on the strain, for an acute fatal-poliomyelitis or a chronic demyelinating disease with persistence of the virus in the central nervous system of the mouse (for review, see refs. 3 and 4).

Classification. The EMCV belongs to the Picornaviridae (pico = small, rna = ribonucleic acid) family, which is currently divided into 12 genera: Aphtovirus, Avihepatovirus, Cardiovirus, Enterovirus, Erbovirus, Hepatovirus, Kobuvirus, Parechovirus, Tescovirus, Tremovirus, Sapelovirus and Senecavirus.[5]

The genus Cardiovirus is divided into two species: the Encephalomyocarditis virus (EMCV) and Theilovirus. The EMCV only harbors one serotype, Mengovirus, being a strain of EMCV. In contrast, Theilovirus comprises Theiler's murine encephalomyelitis virus (TMEV), Vilyuisk human encephalomyelitis virus (VHEV), Thera virus (TRV; isolated from rats), Saffold virus 1–8 (SAFV; isolated from humans, for review about SAFV see ref. 6).[5] TMEV, SAFV and EMCV share about 50% identity.[7-9] Depending on the protein considered, the percentage of identity between TMEV and EMCV can vary from 20 to 70%. The L and 2A, two proteins that are considered as viral security proteins or virulence factor, are the most divergent.[9]

The EMCV characteristics and genome. The EMCV, like other picornaviruses, is a small non-enveloped virus, with an icosahedric capsid of 30 nm diameter[10] and a genome consisting of a positive single-stranded RNA of approximately 7.8 kb that allows direct translation of the RNA into a polyprotein.

The viral RNA is infectious in and of itself, meaning that viral proteins are not required to initiate viral gene expression, and thus its introduction by transfection into cells suffices for production of infectious viral particles. Viral RNA acts as a mRNA during translation and as template during genome replication. The genome has a unique coding region flanked by two untranslated regions (UTR). The 5' UTR is between 800 to 1,200 nucleotides (nt) long, while the 3' UTR is about 120 nt[11] long and composed of short stem-loop structures followed by a poly(A) tail of variable length (20 to 70 nucleotides) (**Fig. 1**).

The viral RNA is thus polyadenylated at the 3' end, but while cellular mRNA are capped with a 7-methyl-guanosine, the 5' extremity of the viral RNA is covalently linked to a 20 amino acid viral protein called VPg (or 3B).[12] Downstream, a poly(C) tract of about 150 nucleotides (depending on the strain) has been found

A

VPg
Poly C
PKs
5'UTR
IRES
AUG
L
VP4
CRE
VP2
P1
VP3
VP1
2A
2B
2B*
P2
2C
3A
3B
3C
P3
3D
3'UTR
AAA n

B

Protein	Properties / domains	Function	Ref

L	• 67 aa / 7.9 KDa
	• zinc finger domain
	• acidic domain
	• 2 phosphorylation sites Thr47, Tyr41
		• Impair nucleo-cytoplasmic traffic:
		  > Nups Phosphorylation (indirectly)
		  > Bind Ran GTPase
		• Necessary to inhibit apoptosis
		• Inhibit stress granule formation induced by TMEV infection
			77, 144, 147-155

VP4	• 70 aa / 7.3 KDa	• Viral capsid protein, myristylated, located entirely on the inner surface of the capsid. Together with VP2 form the VP0 precursor, cleaved during virus maturation.	178
VP2	• 256 aa / 28.6KDa	• Viral capsid protein	11
VP3	• 231 aa / 25.1 KDa	• Viral capsid protein • May be involved in the specificity of encapsidation of neosynthetised viral RNA	73
VP1	• 277 aa / 30.5 KDa	• Viral capsid protein • Interact with cell receptor • Colocalize with LC3 (autophagic marker)	52
2A	• 143aa /16.9 KDa • NLS • NPG(P) • eIF4E binding site	• See figure 3	16,33, 55, 144, 157-162
2B	• 151aa /17KDa • At least one hydrophobic region	• In contrast with PV, EMCV 2B does not inhibit ER-to-Goli protein trafficking • Reduce ER but not Golgi Ca2+ level • Assume to induce membranous rearrangements (with 2C) and formation of membrane vesicles	44, 45
2B*	• 129 aa • transfram protein • The first 12 aa identic to 2B then +2 frame shifting	• Function unknown • Result of a programmed ribosomal frameshifting	17
2C	• 325 aa / 36.5KDa • ATPasic domain • A putative helicase domain (without proven activity)	• ATP ase activity. • Assume to induce (with 2B) membranous rearrengements, formation of membrane vesicles, bind 3'UTR and is implicated in RNA replication). • May be involved in the specificity of encapsidation of neosynthetised viral RNA	175, 73
3A	• 88 aa / 9.9 KDa • Transmembrane protein	• Do not inhibit protein secretory pathway • Localise at the cytoplasmic membranous complex • Colocalise with LC3 (autophagic marker)	47, 48, 52
3B = VPg	• 20 aa / 2.3 KDa	• Covalently linked to 5'end of genomic viral RNA present in the virus particles. • Is assumed to be urydylated (VPg-pUpU) and serve as primer for viral RNA replication.	11
3C	• 205 aa / 21.6 KDa • Cystein protease • LLVRGRTLVV sequence recognized by the ubiquitin ligase	• Protease • Generates mature viral proteins from the precursor polyprotein cleavage sites (indicated by a dark triangle in A) • Ubiquitin ligase substrate (TRIM22, …) • Labile *in vitro* and *in vivo* • Cleave RIG-I	176, 39, 35
3D	• 460 aa / 51.9 KDa • RNA Polymerase	• RNA-dependent RNA polymerase (3D^{pol}) replicates positive and negative viral RNA	179

Figure 1. For figure legend, see page 353.

and is specific to aphtoviruses as well as EMCV. In contrast, TMEV is also a cardiovirus but does not possess this poly(C) tract.[8] Studies with Mengo virus in murine hosts suggest that the poly(C) length may play an important role in viral pathogenesis[13] and will be further discussed in the section "virulence factor."

Adjacent to the poly(C) tract are some pseudoknots with unknown function. The last unit of the 5' UTR is the internal ribosome entry site (IRES). The IRES of picornaviruses fall into five categories, based on conserved primary and secondary structure[14] (for review see ref. 15). EMCV has a type II IRES of about 450 nt with a highly ordered structure made up of hairpin loops. It is subdivided into five structural domains designated H-L, and immediately upstream of the initiation codon AUG a pyrimidine rich tract is found.[16] The IRES allows ribosome binding, and thus the initiation of translation of the open reading frame, which encodes a polyprotein of 2,292 amino acids. Recently, the existence of a programmed ribosomal frameshift has been described, leading to an out-of-frame generation of a cryptic 2B* protein.[17] This finding indicates that the EMCV genome does not have one, but at least two ORF, encoding for 13 mature proteins. The genome of picornaviruses also contains a higher-order RNA structure, the cis-acting replication element (CRE), used as a template for 3D polymerase-mediated VPg uridylation, necessary for initiation of viral RNA replication. In the case of EMCV, the CRE is located in the VP2 coding region and consists of a stem-loop structure with a conserved AAACA sequence in the loop.[18]

Viral proteins. The viral RNA encodes a large polyprotein (L-1ABCD-2ABC-3ABCD) that is cleaved to produce approximately 13 mature proteins. The proteins and precursors of EMCV as well as of the other picornaviruses get their names from their position within the polyprotein, that is, the leader protein (L), the precursor P1, comprising capsid proteins 1A, 1B, 1C and 1D, also called VP4, VP2, VP3 and VP1, respectively, once integrated into the virion. The P2 and P3 are precursors of the non-structural proteins: 2A, 2B and 2C, and 3A, 3B (also called VPg), 2B*, the 3C protease and the RNA-dependent RNA polymerase 3D.[11]

The EMCV genome is represented in **Figure 1** and the accompanying table describes EMCV proteins and summarizes, in their respective order within the polyprotein, their known functions. The function of the EMCV proteins have often been assigned by virtue of their similarity to their well-studied counterparts in poliovirus (PV), Theiler's murine encephalomyelitis virus (TMEV) and foot and mouth disease virus (FMDV). In this review we highlight what has been shown for EMCV and what has been assumed from study of other picornaviruses. (In the next paragraphs we used the term picornaviruses to designate that as far as we know it has not been clearly proven for EMCV but it has been shown for other picornaviruses.)

Three-dimensional structure of the capsid. The Mengovirus has been crystallized and analyzed by X-ray diffraction.[10,19] The viral capsid is organized into an icosahedron with exactly 60 copies of each of the structural protein. The basic unit of the capsid is a protomer formed by the association of VP1, VP2, VP3 and VP4. The VP4 protein is located in the internal face of the capsid and interacts with the viral RNA. Five protomers assemble into a pentamer and 12 pentamers are necessary to form the capsid. The capsid is organized around symmetric axes of 2-, 3- and 5-fold symmetry. Five VP1 proteins are grouped around the 5-fold symmetry axis, while VP2 and VP3 proteins alternate around the 3-fold symmetry axis. In the case of Mengo and TMEV the five copies of VP1 form a plateau with a five branches star shape. The extremities of the branches are constituted of prominent structures (loops) that are the most exposed region at the surface of the Mengo capsid. Each branch of the star is separated by a depression called the pit. The pit corresponds to the contact region between VP1 and VP3. The pit is considered as the analog of the "canyon" found on the capsid of PV. By analogy, it has been supposed that the Mengo pit would serve as a receptor binding site. To support this hypothesis, residues lining in the pit are more conserved than other surface residues[20] and computer modeling can fit a sialic acid residue into a pocket near the bottom of the 22-Å-deep pit.[21]

Viral Cycle

The EMCV viral cycle, represented in **Figure 2**, is detailed below. (The time scale given in the figure legend is specific for BHK-21 and might vary upon the cell type and the EMCV strain use for the infection.)

Adsorption: entry. To initiate the infection, the first step is the attachment of the virus particle to a cell membrane molecule: a receptor or coreceptor.[21] This virus-cell interaction and the distribution of the virus receptor are considered to be determinant for the viral host range, tissue tropism and pathogenesis. Vascular cell adhesion molecule 1 (VCAM-1) is a sialoglycoprotein that has been identified as a receptor for EMCV on murine vascular endothelial cells.[22]

Another 70 KDa sialoglycoprotein found on HeLa and K562 cells and the Sialylated glycophorin A (found on red blood cells) have also been described as being attachment proteins for EMCV.[23,24] Sialoglycoproteins are "sticky" proteins that may have non-specific binding properties. Thus, the main receptor for EMCV, as well as its viral binding partner, remains uncertain. A recent study suggests that the capacity of the virus to interact with cell surface sialic acid residues is important for infection of BRL cells.[25] However, it is interesting to note that sialic acid binding is not required for all EMCV strains.[26]
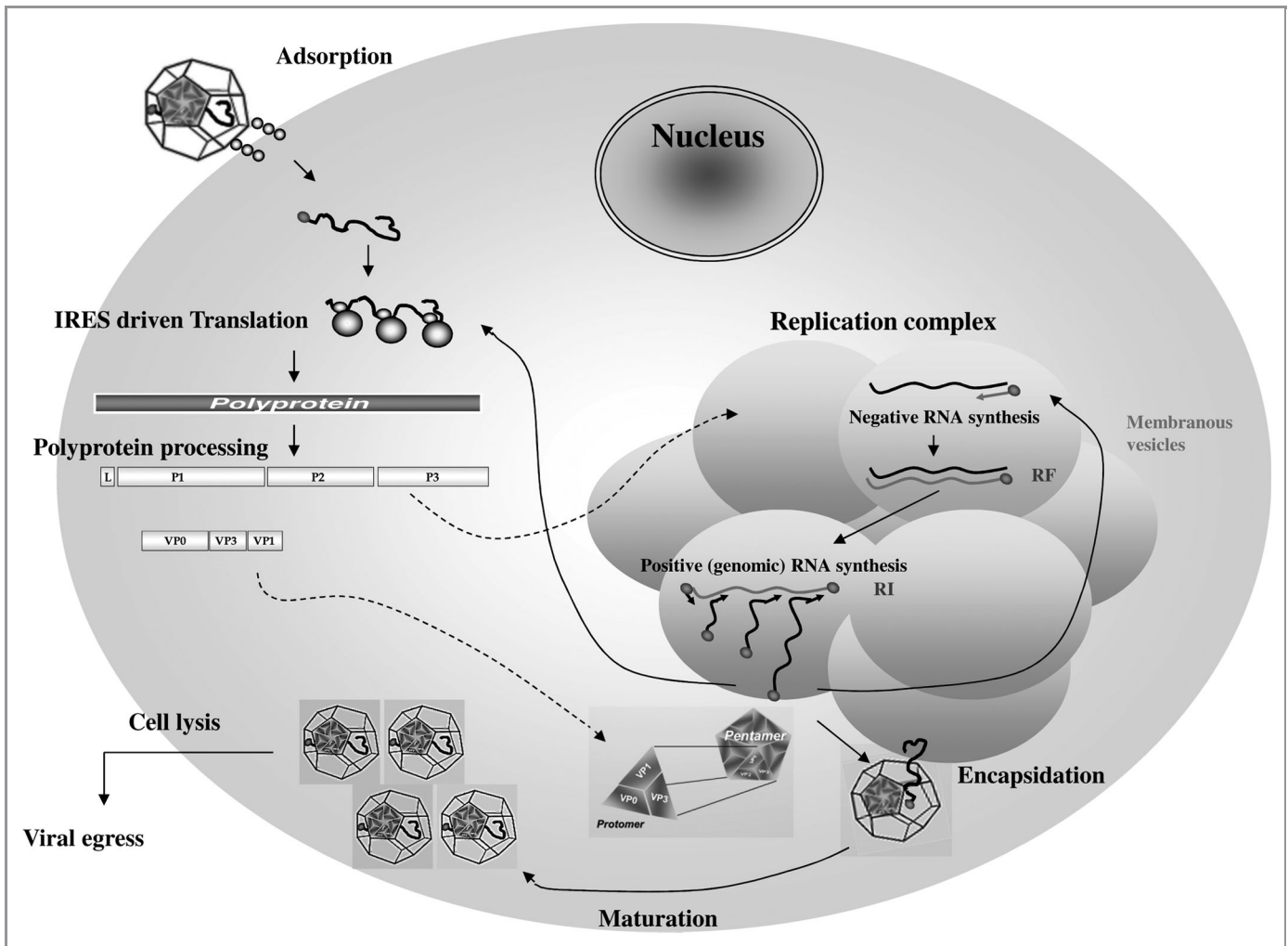
Figure 2. Encephalomyocarditis viral replication cycle. The virion binds to a cellular receptor. After uncoating, the genomic RNA is released by an unknown mechanism. Internalization (~1–2 h). Once in the cytoplasm, the VPg protein is detached to the 5' end of the genome, the translation is initiated at the IRES that require the PTB cellular protein, the polyprotein is synthesized. Translation (~2.5–3 h). The polyprotein is cleaved during and after the translation, leading to precursors or mature proteins. Some of those proteins allow formation of the membranous vesicles where genome replication will occur. The positive genomic RNA is replicated into negative RNA thanks to the VPg-pUpU that serves as a primer for the 3D polymerase. The negative RNA syntesis leads to the production of a double stranded RNA molecule, the replication form (RF). The newly synthesized negative RNA in turn, serves as templates for synthesis of positive RNAs in the replication intermediate (RI). Replication (~3–4 h). Those new positive genomic RNAs will either serve for translation after removal of VPg, serve as matrix for synthesis of new viral RNAs or will be encapsidated. The viral capsid proteins VP0, VP1 and VP3 auto-assemble into a protomer. Five protomers will assemble into pentamers and 12 of them will form the icosahedric capsid. Encapsidation (~4–6 h). After RNA encapsidation, cleavage of the precursor VP0 into VP2 and VP4 allows maturation of the virion. Virions are then egress by cell lysis. Egress (~6–10 h). (Modified from ref. 177).

The mechanisms involved in internalization and uncoating of cardioviruses are poorly documented. It is known that infectivity of EMCV and PV does not require diminution in pH. Thus, unlike FMDV whose entry requires receptor-mediated endocytosis and endosomal acidification, it is believed for EMCV that interaction of the viral particle with a cellular receptor suffices to induce the conformational changes of the capsid shell required for viral RNA delivery into the cytoplasm.[27,28] These conformational changes has been shown for PV to affect contacts between pentamers and thus initiate the dissociation of the capsid. VP1 N-terminal extremities have an amphipathic helix that inserts into the cell membrane and allows the formation of a pore through which the viral RNA is thought to be injected into the cell.[28] However this two-steps dissociation process seems not to fully apply to cardioviruses since contacts between their pentamers are less extensive than for PV.[21] Thus it is still unclear how EMCV release its genome into the cytoplasm.

Translation. Once the genomic RNA is in the cytoplasm, it must be translated as it cannot be replicated by cellular RNA polymerases and no viral enzymes are brought along with the viral particle. The 5' end of the positive single-stranded viral RNA is not capped, but is linked to the VPg viral protein. This VPg does not have any known role in translation and can be detached from the viral RNA by a cellular enzyme.[29] The genome is then

translated into viral proteins needed for genome replication and the production of new viral particles. For EMCV, initiation of translation is cap-independent due to its IRES. There exist five types of IRES that differ in their primary and secondary structures, the location of the initiation codon and their activity in different cell types. All picornaviruses possess an IRES. EMCV and the other cardioviruses have a type II IRES (like the aphtoviruses, but unlike the enteroviruses that has a type I IRES).[30]

The EMCV IRES requires binding of cellular factors, including all the eIFs, except eIF4E, the N-terminal region of eIF4G and the polypyrimidine tract binding protein (PTB).[31,32] This then allows direct recruitment of the 40S ribosomal subunit together with several translation initiation factors (eIF-1A, eIF-GTP-met-tRNA and eIF3), which permits direct initiation of translation at the start codon. Unlike cap-dependent translation, IRES translation does not require eIF4F complex formation; that is,

binding of the eIF4E component to the cap structure at the 5' mRNA and subsequent recruitment of the 40s ribosomal subunit, which scans the 5' non-coding region until the authentic start codon is reached. In both cases, the 60S ribosomal subunit joins the complex at the AUG initiation codon, initiation factors are released, and elongation ensues.

The translation of picornaviral RNA leads to synthesis of viral proteins, some of which inhibit cap-dependent translation. Enterovirus 2A[pro] cleaves eIF4E, a function assumed by the L[pro] for aphtoviruses. The cardioviruses that possess only one protease, 3C, seem to have developed different strategies that enable inhibition of host cell translation, even if this inhibition is slower and less efficient.[33] The EMCV 2A protein is believed to play an essential role in this process (see **Fig. 3** and below the description of "EMCV 2A protein" in the section "EMCV Virulence").
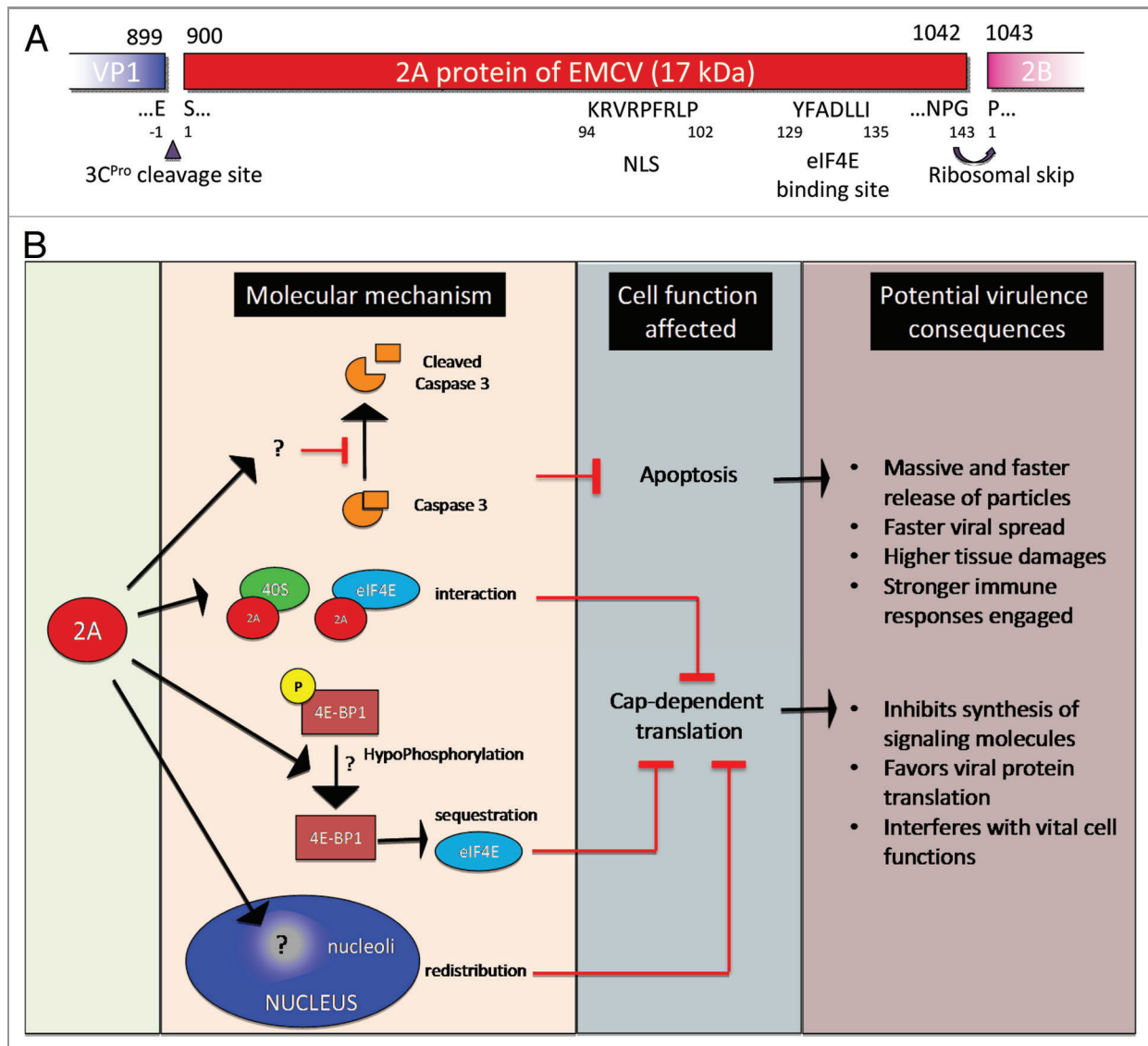

Figure 3. Encephalomyocarditis viral 2A protein. (A) EMCV 2A protein organization and putative sequence. (B) Functionality of EMCV 2A protein and potential consequence on EMCV virulence.

Polyprotein processing. Polyprotein cleavage is mainly performed by the 3C protease (3C^Pro) of EMCV. However, the first instance of polyprotein processing is not proteolytic, and indeed occurs during translation between the 2A and 2B protein, before EMCV 3C^pro synthesis. The NPG(P) sequence at the junction between these two proteins leads to ribosome skipping; no peptide bond is made between the glutamine (G) of 2A and the proline (P) of 2B, resulting in production of two separate peptides (for details see **Fig. 3** and description of the 2A protein in the section "EMCV Virulence").

EMCV 3C^pro, the only protease of EMCV, is an essential protein. It is a cysteine protease that exhibits a high degree of substrate specificity. Comparison of known cleavage sites of the EMCV viral protease has failed to reveal a clear consensus sequence, although some common determinants can be identified. EMCV 3C^pro preferentially cleaves between Q or E and G, S or A residues.[34,35] The precursors 3ABC, 3CD and P3 of EMCV are able to cleave as efficiently as the 3C.[35] Of note, 3CD of EMCV can cleave P1, whereas 3CD of PV cannot.

EMCV 3C^pro has been described to be extremely labile in vitro and in vivo. An LLVRGRTLVV amino acid motif has been identified as a destruction signal that leads to 3C ubiquitination and degradation by the proteasome.[36-38] In contrast to FMDV and PV, only one cellular substrate has been reported for the EMCV 3C^pro. Interestingly, EMCV 3C^pro is able to cleave the RNA recognition sensor RIG-I, at least in vitro.[39] This finding could explain why RIG-I is unable to sense EMCV RNA.

During EMCV infection, once the 3C protease is translated, it is immediately active and starts to cleave the polyprotein in cis. When detached from the polyprotein, 3C^pro can also cleave newly synthesized polyproteins in trans. EMCV 3C^pro cleavage sites within the polyprotein are indicated by small black triangles in **Figure 1**. The last cleavage occurs during the maturation of the virion, after RNA encapsidation, when VP0 is cleaved into VP4 and VP2 (see paragraph "provirion assembly—encapsidation").

Replication. *Localization-formation of the membranous replication complex.* Replication of the picornavirus genome occurs within the cytoplasm. Picornavirus infection leads to intracellular membranous proliferation and rearrangements. The endoplasmic reticulum (ER) and Golgi apparatus are rearranged and the cytoplasm fills up with bilayer membranous vesicles.[40] Viral replication takes place at the vesicle surface.[41] The 3A and 2BC proteins of PV are known to localize in the ER and are sufficient to induce the formation of these vesicles.

The enterovirus 2B protein is a small hydrophobic protein. It is localized in the ER and the Golgi and reduces Ca²⁺ levels in these organelles. The 2B protein is a viroporin,[42] able to form transmembranous pores, which could explain this calcium flux. In addition to membrane rearrangements, it inhibits protein trafficking through the Golgi complex.[43-46] The role of the 2B protein has not been clearly established for EMCV. However, De Jong et al.[44] showed that the 2B protein of EMCV (like that of FMDV) shares little similarity (< 20% sequence identity) with the enterovirus 2B proteins, which are much smaller. The 2B protein of EMCV contains one or more hydrophobic regions, but

no cationic amphipathic α-helix like that found in all enterovirus 2B proteins. Although EMCV 2B, when lowly expressed in cells, does not show a clear ER localization, it was also found to reduce the level of Ca²⁺ in the ER but not the Golgi.[44] The mechanism underlying this decrease is unknown, and the localization of the 2B protein has yet to be confirmed in the context of an EMCV infection. In the same study, the 2B protein of EMCV and FMDV, unlike the 2B protein of enteroviruses, was shown to be unable, on its own, to interfere with protein trafficking.

In addition, the 3A protein, which is a small transmembraneous hydrophobic protein, has also been found in the membranous complex. While the Enterovirus 3A protein inhibits ER-to-Golgi transport, it has been demonstrated that the 3A protein of FMDV, TMEV and EMCV does not.[47,48] Thus, EMCV 3A and EMCV 2B do not block protein transport. It remains to be established whether EMCV has the capacity to block the transport form the ER to the Golgi. Indeed, expression of 2BC or 2B and 2C together are required to block ER-to-Golgi apparatus transport in FMDV infected cells.[49,50] However, it has been described that 2BC of TMEV, when express from a plasmid, does not show the same pattern of localization as 2BC of FMDV.[51] In conclusion, it is yet to be determined whether protein trafficking is inhibited during EMCV infection. To our knowledge, there is no evidence regarding inhibition of ER-to-Golgi protein trafficking in EMCV-infected cells. Of note, even if EMCV does not inhibit ER-to-Golgi trafficking, it induces intracellular membranous proliferation and rearrangements.[52,53]

It is assumed that the lipids that make up the membranous vesicles are derived from the ER.[46] However, those vesicles might also originate from the autophagic cellular pathway. Indeed, during PV infection, it has been shown that the vesicles of the replication complex resemble those of the autophagic pathway, in that they possess double membrane structures, cytoplasmic components within the vesicles, and typical autophagic markers such as LC3 and LAMP1 (latency-associated membrane protein 1).[40,54] Very recently, similar observations have also been described for both EMCV and FMDV. During EMCV infection, colocalization of the 3A and VP1 proteins with LC3 at the surface of double-membrane vesicles has been described.[52] Colocalization of 2B, 2C and 3A viral proteins of FMDV with LC3 and LAMP1, as well as colocalization of VP1 with Atg5 (cellular protein required for autophagosome formation), has been reported.[53] Some studies showed that inhibition of autophagy led to a decrease in EMCV production whereas its induction with rapamycin led to an increase in viral titer.[52,55] This suggests that the autophagic pathway may be hijacked by EMCV, as other picornavirus, to furnish a membranous support for viral replication.[54]

Thus, viral replication of EMCV is believed to occur in the cytosol within the replication complex localized at the surface of clustered membranous vesicles. The replication complex is essentially composed of the viral RNA-dependent RNA polymerase 3D^Pol in association with the 3C^pro, the 2C and the 3AB proteins.

Again, very few studies have addressed the replication of EMCV, and most of our assumptions are based on extrapolation from studies performed with enteroviruses. The model developed

for poliovirus replication is believed to hold for other picornaviruses, but this awaits confirmation.

Negative strand RNA synthesis. It is commonly accepted that translation of picornaviruses must stop to allow transcription of the negative strand viral RNA,[56] negative RNA synthesis being the first step of viral RNA replication. The mechanism governing the shift from translation to replication is not fully determined, although a model has recently been proposed for PV.[57] This model implicates cleavage of the host protein PCBP2 by 3CD[pro]; however, as this cleavage has never been described for EMCV, further studies will be required to decipher how the transition from translation to replication may occur during EMCV infection.

The initiation of negative strand RNA synthesis of picornaviruses is still controversial. A model for PV proposes that such initiation starts with the circularisation of the genome through the interaction of the cellular protein PCBP [poly r(C) binding protein] with the 3CD protein, which itself binds to the cloverleaf structure of the 5'UTR viral genome.[58] For EMCV these interactions might take place at the S fragment or the poly(C). Thus 3' and 5' viral genome extremities may temporarily interact via a protein bridge that forms the so-called ribonucleoproteic complex. The formation of this initiation complex permits the uridylylation of the VPg (3B) by 3D[pol] using the poly(A) tract or the AAACA motif of *cre* as a template.

The *cre* is an RNA stem-loop structure of the viral genome, found in the VP2 coding sequence of EMCV.[18] The loop bears an AAACA, the two first A residues of which acting as a template for the synthesis of VPg-pU and VPg-pUpU (uridylylation) and therefore for the initiation of replication.[59] Mutation within this conserved motif causes a marked decrease in viral replication. Interestingly, the position of the *cre* within the genome seems not to be critical and can even be provided in trans.[18,60] It is believed that VPg-pUpU matches with the poly(A) 3' extremity and therefore acts as a primer for negative RNA strand synthesis by 3D[pol]. The elongation of the negative strand leads to the formation of a double-stranded RNA structure called the replication form (RF).[61] After RF formation, synthesis of new positive RNA can start.

Positive strand RNA synthesis. Picornavirus positive strand synthesis initiation from the RF has not been elucidated. Two main hypotheses have been proposed for VPg-pUpU generation. The first hypothesis suggests that initiation of positive strand synthesis may use VPg proteins that have already been uridylylated, in excess, during negative strand synthesis.[62] The second hypothesis proposes that VPg-pUpU is generated at the poly(A) tail of the positive RNA for the synthesis of the negative strand, while the uridylylation at the *cre* is essentially used for positive strand synthesis.[63]

The RF structure is presumed to be unwound for positive strand synthesis. The 2C viral protein possesses an ATP[ase] activity and a putative helicase domain (even if helicase activity has never been reported), and seems to be important for positive RNA synthesis.[64] It has been reported that the 2C protein binds to the 3' extremity of the negative strand RNA along with the cellular p38 protein. This interaction probably destabilizes the RF.

However, cellular helicases and nuclear proteins might also be implicated, as the RF structure is infectious when used to transfect non-enucleated cells.[61,65]

The negative strand RNA newly synthetized is then used as a template for positive strand RNAs. Indeed, there is formation of partially double-stranded RNA, corresponding to the replication intermediates (RI) that allows simultaneous synthesis of several positive RNA strands from a single negative RNA template.

Provirion assembly: encapsidation, viral release or egress. The last steps in the EMCV viral cycle are encapsidation of newly synthesized viral RNA, maturation of the provirions and virus release from the cell. The mechanisms involved in encapsidation and maturation are still unresolved and are probably the least studied steps of the viral cycle.

The formation of viral particles occurs in the cytosol through the 3C[pro] cleavage of the P1 precursor, releasing VP0, VP1 and VP3 that auto-assemble to form a protomer containing a single copy of each protein. Five protomers form a pentamer and 12 pentamers auto-assemble to form the icosahedric capsid. There are two models for picornavirus RNA encapsidation. A first model proposes that the protomers assemble into empty capsid and that the viral RNA is then inserted into the capsid. This is supported by the observation that empty capsids are found during picornavirus infection, but has never been formally proven. A second model proposes that the pentamers assemble directly around the neo-synthesized viral RNA. Picornaviruses encapsidate only positive single-stranded RNA linked to the VPg protein.[66] In addition, only newly synthesized RNAs are encapsidated, suggesting that a link exists between active replication and encapsidation.[67]

Substitution of P1 by a reporter gene in several picornaviruses does not perturb viral RNA encapsidation when capsid proteins are provided in trans.[68-70] This indicates that the putative encapsidation signal is not found within the P1 region of viral RNA. In fact, numerous attempts to identify an RNA encapsidation signal have failed. A single study reports that the encapsidation signal could lie within the 5'UTR of the Aichi virus together with the L protein.[71,72] However, a recent paper, which tends to favor the second model of encapsidation, also proposes a mechanism by which the triple interaction vRNA-2C-VP3 would be sufficient to explain the specificity of picornavirus encapsidation.[73] The novel mechanism described by Liu et al. suggests that encapsidation takes place at the site where newly synthesized genomes emerge from the replication complex, since morphogenesis is linked to genome replication, 2C ATP[ase] being a component of the replication complex and possessing specific affinity for the VP3 capsid protein. They showed that for chimeric viruses composed of PV and Cox that are unable to support encapsidation, the encapsidation process could be restored if the P1 and 2C proteins originate from the same virus. Previous studies on heterologous encapsidation[68] have shown that the PV replicon could be encapsidated into coxsackie B3 virus (CBV3), human rhinovirus 14 (RHV14) and mengovirus capsids. It should be noted that the capsid proteins were provided by the expression of the full viral cDNA under control of a T7 promoter, indicating that their 2C proteins were certainly present.

The presence of the viral RNA in the capsid is required for the maturation step in which VP0 is cleaved into VP2-VP4. This cleavage is considered to be autocatalytic and would result from a local activation of water molecules by a His residue in the VP2. This would lead to a nucleophilic attack on the scissile bond and cleavage.[74,75] The maturation is necessary for the generation of infectious viral particles. Indeed, it is possible that VP0 cleavage is required for the subsequent release of viral RNA into the cytoplasm of newly infected cells.[76]

EMCV is a rapidly lytic virus that causes necrotic cell death within 7 to 10 h.[77,78] However, just what may lead to membrane permeabilization and what the signals for cell lysis and viral egress may be remain to be characterized. Several hypotheses have been advanced, such as cell burst due to accumulation of viral particles or proteins in the cell or permeabilization of cell membranes due to viroporins (like the 2C of PV). Irrespective of which process is involved, the efficiency of viral release is likely to influence EMCV virulence (further developed in the section on EMCV Virulence).

Host Spectra, Epidemiology and Zoonotic Potential

EMCV was first isolated from a gibbon in 1945 in Florida,[1] and then from swine following an epizootic in Panama in 1958.[79] From 1945 to the present, EMCV has been detected in many wild and domestic animals, in many different areas around the world, such as Europe,[80,81] Canada,[82] South America,[79,83,84] Australia,[85] Korea[86] and China.[87]

A non-exhaustive list of animals from which EMCV has been isolated includes voles, squirrels, elephants, swine, wild boar, racoons, antelope, lions, birds and several species of non-human primates.[1,81,85,88,89] Human infections have also been reported (see paragraph on zoonotic potential).

All together these studies indicate that EMCV has a worldwide distribution and can infect a wide range of animal species. Its natural reservoir is thought to be rodents (mouse or rats). Indeed rat infections are usually asymptomatic and the virus can replicate and be excreted up to 29 d post-infection.[90] The presence of infected rodents in proximity to farms with infected swine also suggests they play an important role in virus spread,[91,92] even more so because horizontal transmission between pigs seems limited.[93] However, a study performed in Greece points out that the possibility of a reservoir existing in wild boar should not be neglected.[88]

EMCV infection occurs by ingestion of EMCV-contaminated food, water and diseased carcasses.[89,94] EMCV is a small non-enveloped virus that is very resistant and may remain infectious for days, even in a hostile environment. EMCV is quite stable in a large spectrum of pH and resistant to ether treatment. Disinfection methods recommended by veterinary are multiples: the virus can be inactivated by treatment at 60°C for 30 min, H_2O containing 0.5 ppm of chlorine, iodine-based disinfectants or mercuric chloride.[95] Recently, a non-invasive and environment-friendly method using a femtosecond laser has been proposed to inactivate the virus.[96] A large scale usage and its extension to other pathogens could be an interesting development for this method.

Zoonotic potential. The EMCV has often been described as a potential zoonotic agent. Nevertheless, an association between human infection and disease has still not been clearly established. Some experimental infections on human explants or human primary cell cultures have been described and clearly point out that human cells are sensitive to EMCV.[97,98]

Between 1940 and 1950 childhood infections associated with fever and encephalitis but no myocarditis were described in Germany and Holland. Several EMCV strains (MM, AK, Li32, Ortilb and SVM) were isolated after inoculation of rodents.[99] However, the assignment of these strains to the EMCV species was determined on the basis of serological tests, without virological confirmation. Unfortunately, these strains are no longer available for further characterization. In an earlier study, neutralizing antibodies against EMCV were found in 17 soldiers based in Manila, Philippines. These soldiers presented febrile illness for 3 d. Three patients out of the four presented an increase in neutralizing antibody titer.[100] In 1948, after isolation of a Mengovirus from a Rhesus monkey, a researcher who was studying the virus and taking care of the animal had developed encephalitis, from which he later recovered. Meanwhile, the virus was isolated from his blood.[101]

From 1950 to 2009, no EMCV infections associated with clinical signs were reported. However, serological studies performed on healthy persons revealed a prevalence of 2.3% to 15%.[102-104] In Austria, a more recent study revealed that 5% of persons with occupational exposure to animals were EMCV seropositive. This percentage reached 15% for hunters.[105,106]

Two recent studies, performed in Peru, described human cases. Patients presented febrile illness, likely due to EMCV infection. Indeed, virus was isolated during the acute phase for two patients with nausea, headache and dyspnea. Upon molecular diagnosis, no virus other than EMCV was detected.[84] Isolation of viruses from sera of acute phase patients supports the idea that EMCV can infect humans and cause disease. A wider study has been performed and indicates that EMCV could be responsible for almost 1% of the febrile illness analyzed during this study in Peru.[83] Despite the low morbidity, human exposure to the virus is quite common. Indeed, 6% to 17% of Peruvian town inhabitants are seropositive for EMCV.[83] These recent data confirm what R. Tesh said in 1978: "EMC infection in man is fairly common but most human cases are probably asymptomatic and/or unrecognized."[102]

EMCV Pathogenesis

EMCV infection occurs by ingestion of EMCV-contaminated food, water and diseased carcasses.[89,94] EMCV is a small non-enveloped virus that is very resistant and may remain infectious for days, even in a hostile environment. There is considerable variation in the severity and location of lesions caused by EMCV in the broad range of susceptible hosts. However, EMCV pathogenesis has mostly been studied in rodents, swine and monkeys.

General symptoms in non-human primates, such as baboons, gibbons, chimpanzees, green and rhesus monkeys, are mainly

death and labored respiration associated with acute heart failure. The salient necropsy findings are pulmonary congestion and edema, hydropericardium, hydrothorax, ascites, lymph node and splenic hypertrophy, and pale white-to-tan mottled hearts. Placental infection with fetal loss can also occur.[107] Sub-cutaneous inoculation of baboons or African green monkeys induces myocardial and nervous disorders leading to death within a week. Infection of monkeys can lead to high mortality in primatology centers, as occurred in a baboon colony in San Antonio in 1992,[107] and more recently in a group of rhesus macaques.[108] Histologically, affected myocardiums present multifocal infiltration by lymphoplasmacytic cells admixed with necrotic and degenerate myofibers and infrequent mineralization.[108]

In pigs. EMCV usually induces acute focal myocarditis with sudden death in pigs. Myocarditis is characterized by cardiac inflammation and cardiomyocyte necrosis. Other symptoms have been observed, such as anorexia, apathy, palsy, paralysis or dyspnea.[109] Susceptible pigs develop severe myocarditis followed by sudden death, while more resistant pigs develop mild myocarditis and can remain asymptomatic. Experimentally infected piglets show high fever, followed by death within 2 to 11 d, but can sometimes recover with chronic myocarditis. Mortality in piglets before weaning can rise to 100%. Mortality decreases with age.[91]

After oro-nasal infection of piglets, viruses are thought to spread from tonsils to target organs by means of infected circulating monocytes.[110] In swine, the heart is considered to be the major target organ.[110] A few days after infection, however, viruses can be isolated from many other organs, such as brain, spleen, intestine, pancreas, liver, kidneys, heart, lungs and lymph nodes.[91,109,111] Upon autopsy of experimentally infected piglets, myocardial lesions, hydropericardium, pulmonary edema, ascites and hydrothorax are observed.[109,110] Hearts are often dilated and show focal areas of necrosis (with uneven greyish white discoloration). The virus can be detected in the myocardium, even when myocardial lesions are small or absent.[109,110]

Histological analysis of piglet hearts reveals myocarditis associated with scattered or localized infiltration and accumulation of mononuclear cells, vascular congestion, edema and myocardial fiber degeneration, with necrosis. In the brain, congestion is accompanied by meningitis, perivascular infiltration of mononuclear cells and neuronal degeneration.[112,113]

Reproductive disorders including abortion, fetal death or mummification have been described in infected females.[114]

In rodents. EMCV infection can be asymptomatic,[90] but in mice generally induces encephalitis,[115] member paralysis,[78,116-118] myocarditis[119] or type 1 diabetes.[120] EMCV infection can also lead to reproductive disorders in pregnant mice,[121] testicular lesions (orchitis) in mice[122] and also to lesions in salivary and lachrymal glands (sialodacryoadenitis) (for review see ref. 123).

Susceptibility to EMCV infection differs according to the strain and age of mice, and the viral strain and dose of inoculum. Infection of mice with different EMCV strains results in a wide spectrum of clinical manifestations, ranging from inapparent infection to severe disease and death.[119] For instance, the D strain of EMCV induces diabetes, whereas the M strain triggers myocarditis.

Myocarditis in mice. EMCV-induced myocarditis is characterized by myocardial necrosis and cellular infiltration. It has been widely used as a model to study viral-induced myocarditis. Many studies seem to indicate that the inflammatory response is deleterious,[124-128] but discordant data do exist.[129] In recent years studies have shown that mast cells also play a critical role in the progression of heart failure induced by EMCV in mice.[124,125] (This will be further discussed in the section dealing with EMCV virulence).

Hind limb paralysis and encephalitis. Hind limb paralysis and encephalitis have been documented during EMCV infection. This indicates that the murine central nervous system (CNS) is attained and damaged during EMCV infection. Upon administration of a high dose of inoculum, mice show encephalitis and paralysis within 4 d of infection and no recovery.[78] Cerebral lesions are mainly perivascular mononuclear infiltrate in the form of perivascular cuffs, multifocal neuronal necrosis and diffuse gliosis[78,117,130] and are predominantly located in the hippocampus.[78,117] EMCV also induces spinal cord lesions comprising focal degeneration, lymphocytic infiltration, mild neuronal necrosis and demyelinisation.[78,118,131,132] Inoculation of a low dose of virus leads to a biphasic hind limb paralysis with possible recovery.[131] EMCV, unlike TMEV and JHL, does not lead to persistent infection of the brain. Hind limb paralysis and demyelinisation occurring during EMCV infection have been proven to be partly due to infiltration of macrophages and lymphocytes[131,132]: treatment of mice with antibodies raised against macrophages, CD4+ and CD8+ T lymphocytes was sufficient to limit the development of the paralytic syndrome and reduced lesions in spinal cords, indicating that these cell types may participate in CNS injury.[133,134] In addition, a study demonstrated that after intracranial inoculation of EMCV, oxidative damage to neurons was due to the induction of NADPH oxidase in microglia (brain macrophages).[135] Thus it seems that inflammatory and immune responses are deleterious for the host CNS tissues.

Diabetes. Infection of genetically susceptible mouse strains with a high dose of EMCV (D) leads to diabetes within 3 to 4 d. This diabetes seems to be mainly due to acute destruction of pancreatic β cells by viral replication without involvement of the immune system. By contrast, during infection with a low dose of EMCV, it was found that the immune system, especially macrophages, plays a central role in the destruction of pancreatic β cells.[136] Inactivation of macrophages prior to viral infection completely prevents EMCV-induced diabetes.[137] It appears that upon low-dose infection of mice the virus initially replicates in pancreatic β cells, leading to the recruitment of macrophages that are activated at the pancreatic islets. These activated macrophages produce soluble mediators like interleukin-1 β (IL-1 β) and produce TNFα and nitric oxide (NO), which induce apoptosis in β cells (for review see ref. 120).

EMCV Virulence

As described in the previous section, EMCV is known to induce myocarditis, diabetes and reproductive and nervous disorders. It is

believed that symptoms and disease are viral strain-specific. Animal species, sex and age are also known to be important factors for EMCV virulence. Few molecular determinants of EMCV virulence have been defined so far. This chapter will describe them and highlight what should be considered to be potential factors in EMCV virulence.

Adsorption and entry. The first primordial steps that can modulate EMCV virulence is the ability of the virus to adsorb to the cell and gain entry. The viral capsid proteins, in their capacity to interact with cellular receptors, are crucial for this entry step and may be considered to be factors that can modulate EMCV virulence. Indeed, mutation of a single amino acid (alanine 776) of the EMCV polyprotein (in the VP1 capsid protein) of a diabetogenic EMCV renders this virus non-diabetogenic. This mutation modifies the native protein conformation of VP1, and thus inhibits virus attachment to the pancreatic β-cell receptor. Indeed, the Ala776 plays a crucial role in promoting viral infection and destruction of β cells, thereby inducing development of diabetes. As regards diabetes induced by EMCV, the genetic background of the mouse is also an important factor.[138] An autosomal recessive gene has been described to modulate the level of expression of the viral receptor at the surface of the pancreatic β cells, and seems to be responsible for mouse susceptibility.[139] In this regard, it has been established that SJL/J mice are susceptible to EMCV-induced diabetes while C57BL/6 mice are not. This discrepancy has most commonly been attributed to a lower level of expression of the viral receptor at the surface of β cells of C57BL/6 mice.[138] Indeed, only certain strains of mice such as SJL/J, SWR/J, DBA/2J and NIH/Swiss develop diabetes, while strains such as C57BL/6J, AKR/J, CBA/J, LP/J and CE/J do not.[138]

Zhu and colleagues recently observed that mutation of the threonine at position 100 of the VP1 protein decreases the neurovirulence of EMCV.[140] This is the first report indicating that a mutation in the viral capsid protein could attenuate its neurovirulence. Substitution of the Thr100 with an isoleucine or a proline reduced the ability of EMCV to grow in the brain of infected mice. The mutants induced only moderate inflammatory responses and less brain damage, but still caused mortality when inoculated at high doses.

The VP1 Thr100 mutation is not as deleterious for EMCV neurovirulence as the mutation in the Ala776, which is determinant for EMCV infection of pancreatic β-cells and gain or loss of diabetogenicity. The role played by the VP1 Thr100 amino acid is unclear and other factors may determine EMCV neurovirulence.

Thus, the VP1 protein of EMCV influences virulent properties, because it is structure by which the virus attaches to its cell receptor and is essential for virus adsorption and entry. However, it should be noted that mutations within viral capsids may also be deleterious for viral assembly as well as release. Indeed, delay in viral release could probably be sufficient to attenuate virulence.[78]

The length of the EMCV poly(C) tract. The length of the EMCV poly(C) tract has been suspected to be important for Cardiovirus virulence. It has been clearly demonstrated that diminution of the length of the Mengovirus poly(C) strikingly attenuated its virulence, rendering it non-pathogenic in mice.[13,141] Of note, a Mengovirus harboring a short poly(C) tract has been investigated as a potentially safe vector for live recombinant vaccines.[141] Studies of different Mengo and EMCV viruses have indicated that virulence of these viruses in mice seems to be positively correlated with their poly(C) tract length, even if short-poly(C) EMCV strains were attenuated only slightly if at all compared with Mengovirus.[142] Thus, a short poly(C) is less deleterious for EMCV strains.[142] Indeed, virulent EMCV strains with very short poly(C) tracts have been described. LaRue et al.[117] isolated an EMCV strain exhibiting a short poly(C) tract (7 to 10 nt) that was pathogenic in mice, pigs and cynomolgus macaques. The fact that a short poly(C) tract is less deleterious for EMCV strains than for the Mengovirus strain indicates that the viral genomic context might influence the degree to which virulence is dependent upon the length of the poly(C) tract.

Viral strain sensitivity to the poly(C) tract length is suspected to be due to a defect in viral replication in certain cell types.[143] How exactly the length of the poly(C) tract may interfere with viral replication remains to be clarified.

2B*. Very recently, Loughran et al.,[17] demonstrated the existence of a programmed ribosomal frameshift that leads to the translation of a cryptic protein of 129 amino acids, called 2B* (represented in **Fig. 1**), which had been previously unknown. These findings indicate that the EMCV genome does not have a single ORF but two and encodes not 12 but rather 13 mature proteins. The 12 N-terminal amino acids of the 2B* protein are identical to those of 2B, and then the +2 frame shift occurs at a conserved GGUUUUU motif. By introduction of synonymous mutations within the conserved motif, the authors showed that inhibition of ribosomal frame shifting and/or absence of 2B* led to a virus with a reduced plaque size phenotype. Importantly, they also observed that efficient frame shifting was only observed in virus-infected cells, indicating that it might be a process "programmed" by the virus. They did not, however, address whether the wt phenotype is restored if ribosomal frameshifting is prevented but expression of the 2B* maintained. It would be of interest to determine whether synthesis of the 2B* protein is important or whether it is the ribosomal frameshifting itself. This process could be important in regulating virus gene expression and may play some sort of regulatory role in the viral lifecycle. It would be interesting to determine whether 2B* plays a critical role in EMCV virulence.

The L protein. The L protein has been termed a "viral security protein" that is nonessential for virus replication but has evolved to fulfil functions that are conserved among picornavirus. The main function of viral security proteins is not to increase virus virulence but rather to counteract host defenses to promote spread within the infected host.[144]

Among picornaviruses that possess an L protein, the L presents considerable diversity as regards both sequence and function (for review, see ref. 144). For instance, the aphtovirus L protein has catalytic activity,[145] while that of cardiovirus does not. Even among cardioviruses there are noticeable differences. The L protein is one of the proteins that displays the greatest divergence between TMEV and EMCV (35 % amino acid identity).[146] The EMCV L protein is 67 amino acids long and contains an atypical

zinc finger and an acidic domain. It can be phosphorylated on residues Thr47[147] and Tyr41,[148] two residues that are not conserved in the TMEV L protein. Moreover, EMCV L lacks the Ser/Thr-rich C-terminal domain present in the TMEV L.

Even if EMCV L protein is devoid of any known enzymatic activity, in contrast to that of aphtovirus, it appears to suppress interferon production[149] and impair nucleo-cytoplasmic trafficking through the binding of Ran-GTPase (Ras-related nuclear -GTPase required for the RNA and protein translocation through the nuclear pores) and through phosphorylation and inactivation of nucleoporins.[150-154]

Substitution of the L protein of TMEV by that of Mengovirus showed that even if these two L proteins display very little similarity (less than 40% identity), they were both able to inhibit transcription of type I interferon, cytokine and chemokine genes, to interfere with nucleocytoplasmic trafficking of host-cell proteins[146] and to inhibit infection-induced stress granule assembly.[155] Stress granules are cytosolic aggregates of stalled translation preinitiation complexes that form in cells exposed to various environmental stresses. They are thought to play a role in translation inhibition during stress, through sequestration of cellular mRNA. When cells recover from stress, stress granules disperse and mRNA can be released for effective translation or targeted to processing bodies (P-bodies). These studies indicate that the L protein counteracts host responses through different pathways, a function that appears to be conserved among different cardiovirus strains and species. Both TMEV and PV have been recently described to inhibit stress granule assembly. However, it is yet to be determined whether EMCV infection induces stress granule formation that is then inhibited by the EMCV L protein. Whether the L protein of EMCV assumes all the functions described when expressed by a TMEV recombinant virus is uncertain. Kinetic analyses of the activity of the EMCV L protein within the context of TMEV recombinant viruses indicate that the L protein of EMCV acts faster than that of TMEV, but that this was deleterious for the recombinant. It should be noted that EMCV replicates faster than TMEV.

This raised the hypothesis that L proteins of TMEV and EMCV diverged during evolution to adapt to the different levels of replication fitness displayed by these viruses.[146]

TMEV strains are divided in two subgroups, GVDII is highly neurovirulent, inducing acute fatal polioencephalomyelitis in mice. The TO subgroup in contrast are strains that induce non-fatal polioencephalomyelitis followed by chronic inflammatory demyelination with virus persistence in the spinal cord (for review, see refs. 3 and 4). It has been shown that strains from the TO subgroup of TMEV possess an out of frame L* which inhibits apoptosis, while the TMEV L present in all subgroup induces it.[156] The L* is believed to play an important role for the viral persistence. In contrast, EMCV that is not known as a persistent virus, has only one L protein, as described above, that has been shown to be required to inhibit apoptosis.

Indeed it has been demonstrated that mutation in the L protein of Mengovirus abrogates its ability to inhibit apoptosis during viral infection.[77] (The inhibition of apoptosis during EMCV infection is further discussed below.)

The EMCV L protein is one of the best characterized proteins of EMCV. It is a multifunctional protein that interferes with many host cell functions. Such interference is likely to be deleterious for the infected cell, and probably for the host, as well. In vivo studies to define whether mutation in the L protein of EMCV would lead to attenuation of virulence are lacking at present. Indeed, an in vivo study would be required to determine unequivocally whether the L protein should be considered to be a virulence factor of EMCV.

The 2A protein, represented in **Figure 3**, is considered to be a second viral security protein of EMCV.[144] It is a small protein of 17kDa, 143 amino acids in length and highly basic, and which, unlike the 2A protein of enterovirus, is not a protease. However, during elongation, prior to the synthesis of the 3C protease, the 2A and 2B proteins are not linked. This interruption in the polyprotein sequence is the consequence of ribosome skipping at the NPG(P) peptide sequence.[157] This interruption, also called "StopGo" translation, is thought to be caused by an interaction between the 2A peptide and the exit tunnel of the ribosome, preventing the interaction of the ribosome with Pro-tRNA (proline is the first amino acid of the 2B protein).

Upon infection with most picornaviruses, host protein synthesis is shut off.[30] This function has been attributed to the 2A protein of EMCV, since partial deletion of this protein result in maintenance of cellular mRNA translation.[55] The shut-off of host protein synthesis that occurs during EMCV infection is not as rapid or extensive as during PV infection.[33] Indeed, in the case of EMCV, it is thought to stem from competition between cap- and IRES-dependent translation, rather than to be a definitive shut-off.[16,158] Several non-exclusive mechanisms involving the 2A protein have been described.

The inhibition of cap-dependent translation could be due to the activation of a translational inhibitor. It has been shown that dephosphorylation of 4E-BP1 triggers the sequestration of eIF4E and inhibits cap-dependent translation. EMCV 2A protein expression in BHK-21 cells triggers 4E-BP1 hypophosphorylation,[55] and thus could contribute to the inhibition of cap-dependent translation. This mechanism, however, has not been confirmed in HeLa and L cell types,[159] where protein synthesis shut-off has also been observed.

Another possibility is that this shut-off is achieved by the interaction of the 2A protein with the translation initiation factor eIF4E. An eIF4E binding site has been located between the amino acids 126–134 of the 2A protein.[159] This interaction might impede eIF4E binding to eIF4G (interactions eIF4E-, eIF4G- and eIF4A are required for initiation of cap-dependent translation). However, alteration of the 2A-eIF4E interaction by introduction of point mutations in the 2A protein is not able to restore normal protein synthesis levels.[159]

Early after EMCV infection, the 2A protein is localized in the nucleoli, owing to its nuclear localization signal (NLS),[159] and associates with the nascent ribosomal subunit.[160,161] Mutations introduced in the NLS have been shown to decrease inhibition of cap-dependent translation. In the cytosol, the 2A protein interacts with the 40S ribosomal subunit.[158,162] This association is believed to induce preferential use of IRES-dependent templates (rather

than capped mRNA). Of note, it has been reported that the 2A protein of EMCV binds RNAs but the functional significance of this interaction remain to be established.

Recently, we have shown that BHK-21 cells infected with EMCV bearing a large deletion in the 2A protein undergo apoptosis through caspase 3 activation, and that induction of apoptosis is independent of the ability of 2A to shut off cap-dependent translation.[78] Thus the 2A protein of EMCV is important in inhibiting apoptosis.

In addition, deletion in the 2A protein profoundly affects EMCV virulence in vitro and in vivo.[55,78] Our study also showed that, despite a large deletion within the 2A protein of EMCV, this virus was still able to infect and replicate in mice. Nevertheless, the virus no longer induced any clinical symptoms, while the wild type virus led to death in all mice within a week. These results were obtained for two different EMCV strains, one that originally induced myocarditis in pigs and another that caused abortion. Thus the EMCV 2A protein is required for viral pathogenesis in mice.

In conclusion, the EMCV 2A protein is important for the virus in counteracting the host defense, as Δ2A viruses do not inhibit cap-dependent translation,[55,159] are unable to inhibit apoptosis in vitro and are no longer pathogenic in vivo.[78] The 2A protein thus well deserves to be considered a virulence factor or a "viral security protein."[144]

Inhibition of apoptosis. The EMCV has mainly been described as a lytic virus that undergoes "necrotic" cell death. Interestingly, observation of viruses bearing modified 2A and L protein (EMCV[78] and Mengo,[77] respectively) has shown that the infection was responsible for apoptotic cell death. These studies suggest that EMCV has evolved to be able to counteract cell host defense through the inhibition of programmed cell death. The exact mechanisms by which the L and 2A protein may interfere with apoptosis remain to be characterized.

Apoptosis is considered to be a common cellular defense mechanism in many viral infections. This process can prevent generation and spread of viral progeny.

Of note, apoptotic cells have been detected during wild-type EMCV infection in vivo.[121,163,164] However, it remains unclear whether apoptosis is a direct or indirect effect of viral infection. As described by Buenz et al.[165] for TMEV-infected mice, the possibility that the majority of cells undergoing apoptosis are uninfected cannot be ruled out. Yamada et al.[164] reported that the main source of apoptotic cells in the heart of EMCV-infected mice appeared to be the infiltrating mononuclear cells and not the cardiac myocytes.

However, more experiments and quantification will be required to decipher whether induction of apoptosis during EMCV infection in vivo results from the virus itself or through the host response to viral infection. In favor to the hypothesis that apoptosis is inhibited during EMCV infection in vivo, it has been shown that inhibition of apoptosis by NFκB is required for EMCV virulence in mice.[166]

Indeed, NFκB, a transcription factor known to induce inflammatory responses and to inhibit apoptosis, is activated during EMCV infection.[167] Matsumory et al.[128] have shown that NFκB activation, by induction of the inflammatory response, is important for EMCV virulence. Thus, inhibition of apoptosis as well as the induction of an inflammatory response might be important factors for EMCV virulence.

Inflammatory response. The inflammatory response induced upon infection plays a critical role in EMCV pathogenesis. As described in the previous chapter, several studies indicate that EMCV neurovirulence might be partly due to CD4^{+} and CD8^{+} T lymphocytes and macrophages.[133-135] Thus, inflammatory and immune responses actually seem to exacerbate EMCV neurovirulence. This may not be peculiar to development of neurovirulence, as it has also been described for diabetes and myocarditis. For diabetes, inoculation of high doses of EMCV induces β cell destruction through direct infection and replication of the virus in these cells. However, using lower doses and despite little EMCV-induced β cell destruction, most damage is actually due to the action of soluble IL-1β and TNFα secretion as well as the production of iNOS from activated macrophages. Indeed, inactivation of macrophages prior to viral infection almost completely prevents EMC virus-induced diabetes.[137]

In the early stages of infection, inflammatory responses are also detected in the heart, where high levels of circulating pro-inflammatory cytokines have been measured.[126,128,168] In the heart of EMCV-infected mice expression of cytokine genes increases, and their degree of expression correlates with the severity of disease.[168] Thus, proinflammatory cytokines may serve to accelerate the pathology of myocarditis, and may have a negative influence on cardiac function.

In recent years, it has been shown that mast cells also play a critical role in the pathogenesis of EMCV induced myocarditis.[124,125,128] Mast cells are powerful producers of multiple cytokines and chemical mediators. Mast cells are considered to be part of the immune system; they present numerous similarities with basophils, but are located in tissues, including the heart. Upon infection of mast cell-deficient mice with EMCV, along with a decrease in production of intracardiac IL-1β, IL-6, TNFα and NO, mice present attenuated myocardial necrosis, diminished cellular infiltration and improved survival rate.[124-128] Although EMCV virulence is attenuated when immune and inflammatory responses are downregulated; no significant effect on viral replication has been noted.[126,128]

Indeed, NFκB is activated during EMCV infections and leads to the expression of many cytokines. Moreover, inhibition of NFκB upon EMCV infection in mice lowered the mortality of the animals, attenuated necrosis and cellular infiltration, and decreased the production of IL-1β, IL-6, TNFα and NO in mouse heart.[126,128] In agreement with these observations, two previous studies indicated that mice lacking the p50 subunit of NFκB more readily survived EMCV infection.[166,169] In addition, it has been reported that the circulating TNFα level is increased in mice infected with EMCV, and that pre-treatment with an anti-TNFα antibody attenuated myocardial injury and decreased mortality in the acute stage.[170] Another group, however, using TNFα knockout mice, reported that TNFα plays a protective role in acute viral myocarditis in mice.[129] These conflicting results might arise from the fact that indeed, an efficient immune system is primordial for elimination of the virus, but an excessive

cytokine storm might be deleterious for the host, as is well-documented in sepsis, for instance.

Altogether, the literature suggests that a strong inflammatory response is of benefit to EMCV virulence. Therefore, one could assume that blocking intracellular protein trafficking would not be advantageous for the virus, since it could decrease cytokine secretion. Indeed, 2B and 3A proteins of PV block ER-to-Golgi trafficking, although the 2B and 3A proteins of EMCV do not.[44,47,48] It has not as yet been determined whether EMCV 2BC, like FMDV 2BC, possesses this ability.[50] It is tempting to suggest that EMCV does not inhibit cell protein trafficking because inflammatory and immune responses are advantageous to its virulence.

The RNA-sensing receptors MDA-5 and RIG-I can be specifically activated by distinct viruses and induce an antiviral response. EMCV exclusively activates MDA-5. Both the EMCV 3C^pro and caspases are supposed to be responsible for the degradation of RIG-I upon EMCV infection.[39] Of note, RIG-I is also cleaved in Poly(I:C) treated cells.[39]

EMCV is able to inhibit regulatory T cell (Treg) function in an antigen presenting cell (APC)-independent manner, but requires that both Treg and effector T cells are simultaneously stimulated through MDA-5.[171] The major function of Treg is to down-regulate the immune response, mainly through the secretion of IL-10, a known anti-inflammatory cytokine. Indeed, it has also been reported that IL-10 expression in EMCV infected mice suppresses the induction of IL-1β and iNOS genes, attenuates myocardial injury and prolongs the survival of mice.[172]

In addition, it has recently been described that the EMCV attachment to the CCR5 receptor suffices to induce production of inflammatory cytokines in macrophages within 30 min. In this study, they made the intriguing observation that the virus does not need to enter the macrophage to promote inflammation.[173] However, they showed that EMCV-infected CCR5-deficient mice produce less iNOS and COX-2, more TNFα and have a 7-fold higher viral RNA load. They propose that the CCR5-induced inflammatory response favors the control of virus spread. No information, however, was provided regarding mouse survival and disease evolution.

All together these data suggest that proinflammatory cytokine production and inflammatory and immune responses are important factors in EMCV virulence in mice. There might be a delicate balance between the beneficial antiviral effect of cytokines and the deleterious effects associated with the cytokine-induced inflammatory responses, which may determine disease development following EMCV infection.

As previously discussed, it has been shown that EMCV induces lytic cell death and inhibits apoptosis, at least in vitro. Yet, it is known that in vivo, lytic cell death gives rise to a greater inflammatory response than apoptosis.[174] The inflammatory response seems to exacerbate EMCV virulence instead of shut it down (discussed above). In addition, inhibition of apoptosis by NFκB has been shown to be required for pathogenesis during EMCV infection in mice.[128,166] It has also been reported that a

virus deleted in 2A, which was no longer able to inhibit apoptosis in vitro, was no longer pathogenic for mice in vivo.[78] Thus, both inhibition of apoptosis and induction of the inflammatory response might be important factors for EMCV virulence. In this study, however, it cannot be ruled out that delay in virus release may be important for virus attenuation. This delay could actually correlate with apoptotic cell death. Indeed, necrotic cell death leads to a massive release of virus that is able to spread and invade the host CNS. In contrast, during apoptosis the cell membranes are not permeabilized, which may constrain virus to confined apoptotic bodies. Apoptotic cells are known to attract phagocytic cells. Thus virus might be retained in these cells and be phagocytosed, without induction of a strong inflammatory response. Thus there might be a link between the defect in virus release, the non-inhibition of apoptosis and the fact that the deleted 2A virus was not able to spread throughout the CNS.

Conclusion

The EMCV is a rapidly lytic virus that does not persist in hosts in most cases. It induces sudden death, myocarditis, encephalitis, nervous disorders and diabetes. Its pathogenicity and the diseases induced depend on viral and host factors. The L and 2A proteins are the two major known virulence factors of EMCV, but understanding of some of their functions at the molecular or host levels is limited. However, EMCV pathogenesis seems to correlate with a strong and uncontrolled inflammatory response that may contribute to EMCV virulence in vivo. Many studies have described an improvement in mouse survival when different components of the immune response are selectively down-modulated. Nevertheless, in other studies the inflammatory response was shown to be protective. This apparent contradiction might reflect a delicate balance between the beneficial effect of the antiviral response and the deleterious effect of a strong inflammatory response. In this balance, inhibition of apoptosis could be one of the major viral activities to tip the scale toward the deleterious side. Indeed, studies using 2A deleted virus suggest a link between viral release, anti-apoptotic activity, inflammatory response and the pathogenicity of EMCV. Further investigations are needed to elucidate how EMCV may manipulate cell death processes to improve viral dissemination, knowledge that could make a major contribution to the understanding of the biology of EMCV.

Although EMCV rarely induces severe clinical symptoms in humans, its worldwide distribution and its wide host range, as well as the biological characteristics of RNA viruses and the use of heart pigs for xenografts, all call for on-going vigilance and improvement of our knowledge of this virus.

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