Immune evasion of feline infectious peritonitis virus-infected monocytes

Els Cornelissen
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Promoter
Prof. Dr. Hans J. Nauwynck

Co-promoter
Dr. ir. Hannah L. Dewerchin
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<th>Description</th>
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<tr>
<td>Abs</td>
<td>antibodies</td>
</tr>
<tr>
<td>ADCC</td>
<td>antibody-dependent cell-mediated cytotoxicity</td>
</tr>
<tr>
<td>ADCML</td>
<td>antibody-dependent complement-mediated lysis</td>
</tr>
<tr>
<td>ADEI</td>
<td>antibody-dependent enhancement of infectivity</td>
</tr>
<tr>
<td>APC</td>
<td>antigen presenting cell</td>
</tr>
<tr>
<td>BCoV</td>
<td>bovine coronavirus</td>
</tr>
<tr>
<td>CCoV</td>
<td>canine coronavirus</td>
</tr>
<tr>
<td>CrFK cells</td>
<td>Crandell feline kidney cells</td>
</tr>
<tr>
<td>CTL</td>
<td>cytotoxic T-lymphocyte</td>
</tr>
<tr>
<td>E protein</td>
<td>envelope protein</td>
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<tr>
<td>EHV 1</td>
<td>equine herpesvirus type 1</td>
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<tr>
<td>FCoV</td>
<td>feline coronavirus</td>
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<tr>
<td>fcwf cells</td>
<td>felis catus whole fetus cells</td>
</tr>
<tr>
<td>FECV</td>
<td>feline enteric coronavirus</td>
</tr>
<tr>
<td>FeLV</td>
<td>feline leukemia virus</td>
</tr>
<tr>
<td>FIP(V)</td>
<td>feline infectious peritonitis (virus)</td>
</tr>
<tr>
<td>FITC</td>
<td>fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FIV</td>
<td>feline immunodeficiency virus</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>granulocyte macrophage-colony stimulating factor</td>
</tr>
<tr>
<td>HCMV</td>
<td>human cytomegalovirus</td>
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<tr>
<td>hpi</td>
<td>hours post inoculation</td>
</tr>
<tr>
<td>HSV</td>
<td>herpes simplex virus</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>Ig G, A</td>
<td>immunoglobulin G, A</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ILLs</td>
<td>innate-like lymphocytes</td>
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<tr>
<td>IPMA</td>
<td>immune peroxidase monolayer assay</td>
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<tr>
<td>iTreg cells</td>
<td>induced regulatory T cells</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
<td>-----------</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Dalton</td>
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<tr>
<td>M protein</td>
<td>membrane protein</td>
</tr>
<tr>
<td>mAbs</td>
<td>monoclonal antibodies</td>
</tr>
<tr>
<td>MHC</td>
<td>major histocompatibility class</td>
</tr>
<tr>
<td>MHV</td>
<td>mouse hepatitis virus</td>
</tr>
<tr>
<td>moi</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>N protein</td>
<td>nucleocapsid protein</td>
</tr>
<tr>
<td>NK cell</td>
<td>natural killer cell</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>O2</td>
<td>superoxide ions</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>pAbs</td>
<td>polyclonal antibodies</td>
</tr>
<tr>
<td>PAMP</td>
<td>pathogen-associated molecular pattern</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
</tr>
<tr>
<td>PRR</td>
<td>patterns recognition receptor</td>
</tr>
<tr>
<td>PRV</td>
<td>pseudorabies virus</td>
</tr>
<tr>
<td>S protein</td>
<td>spike protein</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen free</td>
</tr>
<tr>
<td>TCID&lt;sub&gt;50&lt;/sub&gt;</td>
<td>tissue culture infective dose with a 50% endpoint</td>
</tr>
<tr>
<td>TGEV</td>
<td>transmissible gastroenteritis virus</td>
</tr>
<tr>
<td>TGF</td>
<td>transforming growth factor</td>
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<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
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</table>
Chapter 1

Introduction
1.1 Feline coronaviruses

The disease feline infectious peritonitis (FIP) was presumably first described in 1912 and years later more cases followed (Holzworth 1963; Feldmann and Jortner, 1964). A virus was identified as the etiologic agent and in 1979 it was classified as a coronavirus, labeled feline infectious peritonitis virus (FIPV) (Zook et al, 1968; Ward et al, 1968; O’Reilly et al, 1979). A second feline coronavirus was described in 1981, the feline enteric coronavirus (FECV) (Pedersen et al, 1981). In the beginning, the two viruses were seen as different virus species of the feline coronavirus (FCoV) but are now determined as virus virulence variants, biotypes or pathotypes of the same virus.

1.1.1 Classification, biotypes and serotypes

FCoV belongs to the genus Coronavirus which, together with the genus Torovirus, form the Coronaviridae family. This family belongs together with the Arteriviridae and the Roniviridae to the order of the Nidovirales. The taxonomy of the Nidovirales and coronaviruses of different vertebrates are given in Table 1.1. The genus Coronavirus is divided in 3 groups (1 to 3) based on serological, genetic and antigenic properties (González et al, 2003). FCoV belongs to group 1 and is closely related to canine coronavirus (CCoV) and transmissible gastroenteritis virus (TGEV).
### Table 1.1: Nidovirales taxonomy and virus strains in the genus Coronavirus.

#### Order of the Nidovirales

- Family *Arteriviridae*
- Family *Roniviridae*
- Family *Coronaviridae* *
  - Genus Torovirus
  - Genus Coronavirus

<table>
<thead>
<tr>
<th>Group</th>
<th>Virus Strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Canine coronavirus (CCoV)</td>
</tr>
<tr>
<td></td>
<td>Feline coronavirus (FCoV)</td>
</tr>
<tr>
<td></td>
<td>Human coronavirus (HCoV) 229E and NL63</td>
</tr>
<tr>
<td></td>
<td>Porcine epidemic diarrhea virus (PEDV)</td>
</tr>
<tr>
<td></td>
<td>Porcine respiratory coronavirus (PRCoV)</td>
</tr>
<tr>
<td></td>
<td>Transmissible gastroenteritis virus (TGEV)</td>
</tr>
<tr>
<td></td>
<td>Ferret enteric coronavirus (FrECV)</td>
</tr>
<tr>
<td></td>
<td>Bat coronavirus (BatCoV) 1A, 1B, KHU7 and HKU8</td>
</tr>
<tr>
<td>2a</td>
<td>Bovine coronavirus (BCoV)</td>
</tr>
<tr>
<td></td>
<td>Human coronavirus (HCV) OC43 and HKU1</td>
</tr>
<tr>
<td></td>
<td>Human enteric coronavirus (HECoV)</td>
</tr>
<tr>
<td></td>
<td>Mouse hepatitis virus (MHV)</td>
</tr>
<tr>
<td></td>
<td>Porcine hemagglutinating encephalomyelitis virus (PHEV)</td>
</tr>
<tr>
<td></td>
<td>Canine respiratory coronavirus (CRCoV)</td>
</tr>
<tr>
<td></td>
<td>Rat coronavirus (RCoV)</td>
</tr>
<tr>
<td></td>
<td>Equine coronavirus (ECoV)</td>
</tr>
<tr>
<td></td>
<td>Turkey coronavirus (TCoV)</td>
</tr>
<tr>
<td>2b</td>
<td>Severe acute respiratory syndrome coronavirus (SARS-CoV)</td>
</tr>
<tr>
<td></td>
<td>Bat SARS coronavirus (Bat-SARS-CoV)</td>
</tr>
<tr>
<td>3</td>
<td>Avian infectious bronchitis virus (IBV)</td>
</tr>
<tr>
<td></td>
<td>Pheasant coronavirus (PCoV)</td>
</tr>
<tr>
<td></td>
<td>Goose coronavirus (GCoV)</td>
</tr>
<tr>
<td></td>
<td>Duck coronavirus (DCoV)</td>
</tr>
<tr>
<td></td>
<td>Pigeon coronavirus (PiCoV)</td>
</tr>
</tbody>
</table>

* A proposal for a new *Coronaviridae* taxonomy has been submitted to the International Committee on Taxonomy of Viruses (ICTV), based on rooted phylogeny and comparative sequence analysis of *Coronaviridae*-wide conserved domains. The genera Coronavirus and Torovirus convert to two sub-families *Coronavirinae* and *Torovirinae* respectively. Within the *Coronavirinae* the phylogroup 1 through 3 convert into genera designated *Alpha*-*, Beta*- and *Gammacoronavirus* respectively. Within the genus *Alphacoronavirus*, 8 distinct species have been suggested. The FCoV belong together with TGEV and CCoV to the species Geselavirus (gene seven last).
FCoV infection can occur in two virulence variants, also called *biotypes* or pathotypes. FCoV biotypes that cause only mild, often subclinical enteritis are designated avirulent FCoV or feline enteric coronaviruses (FECV). Biotypes that cause FIP, a highly lethal systemic infection, are designated virulent FCoV or feline infectious peritonitis viruses (FIPV). In this thesis the terminology of FCoV will be used for general characteristics. The terminology FECV and FIPV will be used for characteristics typical for avirulent and virulent FCoV strains respectively. Comparative sequence analysis showed that FIPV arises from FECV by mutation (Poland et al, 1996; Vennema et al, 1998). The mutation that causes the change in virulence has been variably ascribed to deletions in the open reading frames 3c, 7a or 7b or to differences in the S protein (Vennema et al, 1998; Kennedy et al, 2001; Rottier et al, 2005). No morphological or antigenic differences were detected between FECV and FIPV (Pedersen et al, 1981). More than 98% of the genome is identical in FIPV and FECV isolates from the same environment (Vennema et al, 1995).

To complicate the situation, virus-neutralization assays revealed that FCoV occurs in two *serotypes* (I and II). The antibodies against one serotype can only neutralize strains from the same serotype (Pedersen et al, 1983). Serotype II strains are double recombinants of serotype I strains and canine coronavirus (Herrewegh et al, 1998; Vennema, 1999). As a result of this recombination, serotype II strains possess a canine coronavirus spike protein that differs from the feline spike protein of serotype I. The serotype I strains prevail in the field, causing 70-95% of the infections (Hohdatsu et al, 1992; Kennedy et al, 2002; Addie et al, 2003, Benetka et al, 2004; Kummrow et al, 2005; Lin et al, 2008). But serotype I strains are less frequently used in research, due to their low ability to grow in cell cultures.

**1.1.2 Virus structure**

FCoV particles are spherical with a diameter of 60-120nm. The positive single stranded RNA is bound by nucleocapsid (N) proteins and together they form a helical capsid. This capsid is surrounded by a lipid bilayer, the envelope. Within this envelope, three membrane proteins are embedded: the spike (S) glycoproteins, the
membrane (M) proteins and the small envelope (E) proteins. An illustration is given in Figure 1.1.

![Diagram of a FCoV virion](image)

**Figure 1.1:** A schematic representation of a FCoV virion. The helical capsid consists of positive single stranded RNA bound by nucleocapsid (N) proteins. This is surrounded by an envelope containing three membrane proteins: the spike (S) glycoproteins, the membrane (M) proteins and the envelope (E) proteins.

The coronavirus genome (approximately 30 kilobases) is the largest known non-fragmented viral RNA genome. The genes are organized in a 3’ nested set of subgenomic mRNAs, which is typical for the Nidoviruses.

More than two thirds of the genome consists of two large open reading frames (ORF1a and 1b) encoding two polymerase polyproteins (1a and b). These polyproteins are proteolytically processed by viral proteases to generate proteins for viral RNA replication and transcription. The downstream ORFs encode for the structural proteins and some accessory proteins.

The S glycoproteins are 150 kiloDalton (kDa) with the majority of the protein present outside the viral envelope and a small cytoplasmic tail. These petal shaped S proteins protruding from the viral envelope give a corona (crown)-like appearance in electron microscopic pictures. S proteins play a role in virus-receptor binding, virus-cell and cell-cell fusion and induction of neutralizing antibodies (de Groot et al, 1989).
The E proteins (10 kDa) are almost entirely embedded in the envelope with only a short cytoplasmic tail (Maeda et al, 2001). These proteins are necessary for envelope formation and capture the other membrane proteins at the budding site (Lim and Liu, 2001).

The M proteins (25-30 kDa) are the major component of the viral envelope with only 10% outside the viral envelope. The M proteins mediate assembly of the virion via interactions with the N proteins (Holmes, 1985; Spaan et al, 1988; Lai, 1990). They may also play a role in viral entry since neutralizing antibodies against M proteins have been described (Vennema et al, 1991; Kida et al, 2000).

The N proteins (43-50 kDa) bind to the RNA strand and form the helical nucleocapsid. Through interactions between the N and M protein this nucleocapsid is incorporated into newly formed virions (Narayanan et al, 2000). The N protein plays also a role in replication and/or transcription (Spaan et al, 1988).

The viral genome encodes also for proteins that are not necessary for virus growth and infection and are therefore called accessory or group specific proteins. For FCoV the accessory genes are encoded by ORF 3abc and 7ab. The 7b protein (26.5 kDa) is a soluble protein that is secreted from infected cells and might act as an immune modulator (Vennema et al, 1992; Rottier, 1999). The precise function of these accessory proteins is not known but deletion of one or both of these gene clusters has an attenuating effect on the virus (Haijema et al, 2004). Also, a mutation in 3c, 7a or 7b has been correlated with development of FIPV strains from FECV strains (Vennema et al, 1998; Kennedy et al, 2001).

1.1.3 Host range and epidemiology

FCoV-infections occur in different members of the Felidae family. Both domestic cats and non-domestic cats like cheetahs, (mountain) lions, European wildcats and cougars can be infected (Heeney et al, 1990; Paul-Murphy et al, 1994; Hofmann-Lehmann et al, 1996; Leutenegger et al, 1999; Biek et al, 2006). FCoV is endemic within the population of the domestic cats, especially in multicat households or breeding facilities with shared litter-, food- and drink trays (Pedersen, 1995). Up to 90% of these cats are seropositive for FCoV, compared to 10-60% of single household cats and 15% of stray cats (Horzinek and Osterhaus, 1979; Sparkes et al,
1992; Pedersen et al, 2004). The vast majority of this FCoV seropositivity is caused by FECV. In most cases, cats become infected with FECV when maternal immunity wanes (Pedersen et al, 1981; Addie and Jarrett, 1992).

Although the prevalence of FCoV infection is high, the number of FIP cases is low and rarely passes 5% of the seropositive cats in multicat households. This number is even lower in single cat household (Addie and Jarrett, 1992; Addie et al, 1995; Pedersen, 1995). The highest prevalence of FIP is seen in young cats of 6 months to 2 years of age (Addie and Jarrett, 1992). FIP is reported more frequently in pure breed cats. This can be due to the fact that these cats are often kept in multicat households or due to enhanced genetic susceptibility of some highly inbred population of cats (Rohrbach et al, 2001; Pesteau-Somogyi et al, 2006). Other factors associated with increased FIP prevalence are high FCoV antibody titers, an increase in factors associated with ‘stress’, regular introduction of new cats to a cattery and the presence and proportion of chronic coronavirus shedders (Kass and Dent, 1995; Foley et al, 1997a; Pesteau-Somogyi et al, 2006).

1.1.4 Pathogenesis and clinical signs

FECV

Transmission of FECV occurs via the fecal-oral route. After digestion, the virus primarily infects enterocytes in the small intestines, especially the duodenum, jejunum and ileum. The virus binds to the apical side of the villi, enters the enterocytes and due to replication, the enterocytes are destroyed. FECV can also spread through the body, probably via blood-born monocytes, and reach different organs like mesenterial lymph nodes, tonsils, thymus, spleen and bone marrow (Pedersen et al, 1984; Herrewegh et al, 1995; Gunn-Moore et al, 1998; Meli et al, 2004). Most FECV infected cats are asymptomatic. A transient and clinically mild diarrhea and vomiting can occur as a result of the replication in the enterocytes, especially in kittens (Pedersen et al, 1981). Rarely, the virus may cause severe acute or chronic vomiting and/or diarrhea which can be fatal (Kipar et al, 1998a). No systemic effect has been detected with FECV.

Viral excretion in the faeces starts within a week with a peak approximately 7 days after infection (Pedersen et al, 1984). It can continue during a long period (2-10
months) which is followed by persistent excretion, intermittent/recurrent shedding or ceasing of shedding (Pedersen et al, 2008). Viral excretion is significantly higher in kittens and seems to be higher in cats with higher FCoV antibody titers (Foley et al, 1997b; Pedersen et al, 2008). Chronic asymptomatic carriers show viral persistence in the lower part of the intestinal tract and probably shed virus for the rest of their lives (Herrewegh et al, 1997). The factors that cause a cat to become a carrier or to be transiently infected are unclear. Both host and viral factors can play a role. In most studied cases it was independent of the virus strains, so it appears that the determining factors are mainly a property of the cat rather than a property of the virus strain (Addie et al, 2003).

In general, there are 4 outcomes of an FECV infection (Addie et al, 2004a,b).

(i) The immune system reacts and the cat overcomes the infection. In time, the cats become susceptible again for new FECV infections (70-80%).

(ii) The cat becomes a lifetime asymptomatic carrier and shedder (10-15%).

(iii) The cat is resistant to infection with FCoV (less than 5%).

(iv) The virus mutates and the cat develops FIP (less than 5%).

**FIPV**

The current belief is that FIPV, unlike FECV, is not transmitted from cat to cat but emerges within the cat in which it causes disease (Addie et al, 2004b; Vennema et al, 1998). It is hypothesized that the location where FECV mutates into FIPV is most probably the intestines and not the monocytes, based on the fact that FECV replication in monocytes is very limited, while in enterocytes a high replication and thus a higher mutation rate is present (Stoddart and Scott, 1989; Rottier et al, 2005). Each factor that enhances FECV replication in the intestines enhances the chance of mutation into FIPV.

It is not clear why the mutated viruses have the potential to induce FIP and if viral factor and/or host factors are important. It is now speculated that FIPV is capable of infecting monocytes more effectively, due to mutations in the S protein (Stoddart and Scott, 1989; Rottier et al, 2005). This higher replication can lead to a high level of monocyte-associated viremia and higher viral load which could be a key factor in the development of FIP. On the other hand, higher viral loads can also be due to
lower viral clearance in cats developing FIP, meaning that individual host factors can play a role, particularly the immune responses (de Groot-Mijnes et al, 2005; Kipar et al, 2006a).

The activated monocytes disseminate the virus and initiate the development of *granulomatous vasculitis* (Kipar et al, 2005). The monocytes adhere to and can migrate through small and medium-sized veins. This leads to circular or focal infiltrates in the vascular wall or perivenous cellular agglomerates. Besides the granulomatous vasculitis, demarcated or confluent *granulomas* (with or without necrosis) are formed in various organs. In all lesions, the most predominant cells are monocytes/macrophages (of which variable numbers are virus positive) and a variable amount of T lymphocytes, B lymphocytes, plasma cells and neutrophils (Kipar et al, 1998b; Paltrinieri et al, 2001; Berg et al, 2005; Kipar et al, 2005). Complement factor 3 and antibodies have been detected in these lesions, indicating that they may play a role in further development of the vasculitis and granulomas (Pedersen and Boyle, 1980). Immune-complex formation and deposition and complement activation could also contribute further to the pathogenesis (Jacobse-Geels et al, 1980, 1982). Due to the vasculitis and the formation of granulomas, protein-rich fluid (exudate) can leak into the body cavities.

Clinical signs of FIP are variable because many organs may be involved. They are a direct consequence of the vasculitis, granulomatous lesions and organ failure resulting from the damage to the blood vessels (Olsen, 1993). There are two clinical forms of FIP: an effusive, wet form and a non-effusive, dry form. A combination of both can occur. The first clinical signs are similar in both forms and consist of anorexia, weight loss and a chronic fluctuating fever that is antibiotic resistant (Olsen, 1993; de Groot and Horzinek, 1995). The wet form is characterized by effusions present in one or more body cavities like abdominal or thoracic cavity or in the pericardium. This can give a non-painful enlargement of the abdomen, dyspnea and heart insufficiency respectively. Multiple granulomas are formed on the serosae of different organs and fibrin can be present (Weiss and Scott, 1981a). Cats with the wet form of FIP deteriorate quickly and will die after weeks or a couple of months (Olsen, 1993). In all cats, the disease progression of FIP is multiphasic (Weiss and Scott, 1981a; de Groot-Mijnes et al, 2005). It starts with a phase of enhanced viral replication coinciding with fever, loss of body weight, jaundice, T-
cell depletion and detectable viral RNA in the blood. This is followed (day 7 to 8) by a phase of recovery characterized by normal body temperature, stabilization or increase in body weight and no detectable RNA levels present in the blood. The further outcome of the infection varies from cat to cat. Most cats (74%) succumb during the following phase of viral replication within 36 days. Others can survive up to 2 relapses (survival time 50-54 days). Long term (> 100 days) survivors (19%) can apparently succeed in controlling the virus after 1 to 3 phases of viral replication and are free of lesions (de Groot-Mijnes et al, 2005).

In the dry form, little or no effusion is present. Larger granulomas are formed, especially in the kidneys, the mesenteric lymph nodes and the liver (Pedersen, 1987). If organs are severely damaged, clinical signs of liver or kidney insufficiency can be present. Central nervous symptoms, like ataxia, paresis, nystagmus, behavioral changes, incoordination or seizures, can also be observed. Ocular symptoms can be present like retinal changes, iritis or uveitis (Andrew, 2000). Cats with the dry form of FIP can survive over a period of many months.

Illustrations of clinical signs and pathological findings seen during effusive and non-effusive FIP are given in Figure 1.2.
Introduction

<table>
<thead>
<tr>
<th>Effusive/wet form</th>
<th>Non-effusive/dry form</th>
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<tbody>
<tr>
<td><img src="image1.png" alt="Image a" /></td>
<td><img src="image2.png" alt="Image e" /></td>
</tr>
<tr>
<td><img src="image3.png" alt="Image b" /></td>
<td><img src="image4.png" alt="Image f" /></td>
</tr>
<tr>
<td><img src="image5.png" alt="Image c" /></td>
<td><img src="image6.png" alt="Image g" /></td>
</tr>
<tr>
<td><img src="image7.png" alt="Image d" /></td>
<td><img src="image8.png" alt="Image h" /></td>
</tr>
</tbody>
</table>

1. **Clinical signs**

2. **Pathological findings**

Figure 1.2: Possible clinical signs and pathological findings seen during FIP. Enlargement of the abdomen (a, b) due to formation of exudate in the abdominal cavity (c) with formation of multiple small granulomas on the intestines (d) observed during the effusive form. Ocular changes (e) with protein precipitates (f) and formation of larger granulomas on the kidney (g, h) observed during the non-effusive form.
1.1.5 Diagnosis

FECV

The clinical importance for diagnosing FECV is very low. When intestinal clinical signs occur, they cannot be distinguished from other infectious and non-infectious causes. A possible correlation with FECV replication can be determined by electron microscopical demonstration of coronavirus particles in the faeces (Dea et al, 1982; Kipar et al, 1998a). Virus isolation from faeces is also possible, but has a higher risk of false negative result due to the low growth ability of serotype I strains in cell cultures. Post-mortem diagnosis can demonstrate coronavirus antigen within intestinal epithelial cells (Kipar et al, 1998a). Serial fecal PCR-test can be used to identify chronic shedders in a multi-cat environment, which can be important in limiting virus circulation (Foley et al, 1997b).

FIPV

FIP is a difficult condition to diagnose, many other conditions present with very similar clinical signs and hematological and biochemical results (Sparkes et al, 1991). The diagnosis of FIP is a probability diagnosis: the more clinical signs and laboratory results can be due to FIP, the higher the probability for FIP (Rohrer et al, 1993). Blood tests can show a lymphopenia, anemia, neutrophilia, higher bilirubin, AST and α1-acid glycoprotein (AGP) levels and a hyperglobulinemia resulting in a lower albumin/globulin ratio. Tests of the exudate show a high total protein level (over 35g/l), a lower albumin/globulin ratio, a high AGP level and presence of mainly neutrophils and macrophages. The presence of antibodies in the blood or exudate only indicates that the cat has been infected with FCoV. Any FCoV antibody titer can occur in cases of wet or effusive FIP, but most cats with dry FIP have high antibody titers (Addie, 1989). Antibody titers of 0 are unusual in FIP cases but are possible (Barlough, 1985). Diagnostic tests, like antibody titer and protein tests, that can be performed on exudate have higher predictive values than tests performed in blood (Hartmann et al, 2003). Definitive diagnosis is only possible when coronavirus antigens can be detected in monocytes/macrophages in granulomas or exudate by immunofluorescence (illustrated in Figure 1.3) or immunohistochemistry (Parodi et al, 1993; Tammer et al, 1995). This is possible in
collected exudate or in biopsy from living cats. Unfortunately, macrophages cannot be detected in all FIP induced effusions and biopsy is an invasive, expensive method with also false negative results (Hartmann et al, 2003; Giordano et al, 2005). In most cases the definitive diagnosis is made post mortem.

Figure 1.3: Diagnosis of FIP by visualizing feline coronavirus antigens (green signal) in a granuloma using immunofluorescence staining. Mononuclear cells bearing FCoV antigens in their cytoplasm are detected (arrows).

1.1.6 Treatment and prevention

FECV

In general, treatment of a FECV infection is not necessary, due to its mild and self limiting character. Fluid and electrolyte therapy can be required if strong gastro-intestinal clinical signs are present.

Prevention of FECV also seems unnecessary because of its mild character. Though, if FECV infection and spread could be limited or prevented, the mutation rate to FIPV would effectively be reduced or inhibited. Management plays a crucial role in limiting virus spread, minimizing exposure and eliminating stress. Important is to minimize faecal contamination by placing litter boxes away from food trays, regularly cleaning of the litter boxes and housing. A stable group of cats with limited number of cats, limited introduction of new cats and limited continuous presence of kittens is also recommended (Pedersen et al, 1995).

Total elimination of FECV in multi-cat household is possible. It can occur spontaneously and naturally in households with less than 5 cats (Gonon et al, 1995). In households with more cats, multiple measures have to be taken to eliminate
FCoV. These includes: identifying and removing continuous shedders, quarantine of seropositive animals until they become seronegative and disinfection of all possible contaminated surfaces to prevent fecal-oral transmission. These measures can be time-consuming, costly and must be followed rigorously (Hickman et al, 1995). Development of vaccines against FECV has not been assayed. It is doubtful that a successful vaccine against FECV can ever be developed, since no vaccine can work better than a natural infection which in itself does not result in long-term protection (Addie et al, 2004b).

**FIPV**

If FECV mutates to FIPV with development of clinical signs, no effective treatment is available and over 80-95% of the cats will die (Addie et al, 2004b; de Groot-Mijnes et al, 2004). Treatment can be beneficial in early stages and can slow down the progression of the disease and prolong the cat’s life. This treatment is mainly aimed at modulating the immune response triggered by the infection. The most used treatment consists of relatively high doses of immunosuppressive and anti-inflammatory drugs. Therefore, glucocorticoids are used, sometimes in combination with cytotoxic drugs or interferons (Bölcskei and Bilkei, 1995). The efficiency of these treatments is difficult to evaluate since well-controlled clinical trials are rare (reviewed by Hartmann and Ritz, 2008). For example, recombinant feline interferon omega seemed to have a possible positive effect in FIP treatment in a not-controlled study, whereas in a placebo-controlled double blind study no effect was seen (Ishida et al, 2004; Ritz et al, 2007).

Vaccines against FIPV have been extensively studied. Most vaccine strategies were unsuccessful since they did not show (significant or consistent) protection or they even gave enhancement of the disease (reviewed by de Groot and Horzinek, 1995). The only vaccine that is commercially available against FIP is a temperature-sensitive strain of a serotype II FIPV. This can be administered from 16 weeks of age and has an efficacy of 50-75% in seronegative kittens (Gerber, 1995; Postorino Reeves, 1995). When cats already have been in contact with FCoV and consequently have antibodies, the vaccine is ineffective (Fehr et al, 1995). Thus, vaccination in an FCoV endemic environment will be ineffective since cats will become infected...
before the time of vaccination (16 weeks). More recently, a promising vaccination approach using a live attenuated serotype II strain with deletion of the accessory gene cluster 3abc or 7ab was described (Haijema et al, 2004). Protection against a homologous serotype II challenge was very high, 80 and 100 % for the 7ab and 3abc respectively. It remains to be elucidated if these serotype II vaccines provide protection against a serotype I challenge.

1.2 Immune response and immune evasion

In the following paragraphs an overview is given of possible immune responses against viruses (detected in humans or other mammals) and viral immune-evasion mechanisms (1.2.1). This is followed by the limited information known about the immune response against FCoV (1.2.2). In general, there is less known about the immune system of the cat than that of humans, mice or other domestic animals. The components of the immune sytem which have been detected in cats show similar biological properties with other animal species (Lin, 1992).

1.2.1 In general

Pathogens have a broad range of mechanisms to infect their host. Therefore, it is not surprising that the immune response uses a complex variety of protective mechanisms to control and eliminate these pathogens. The immune response consists of an innate and an adaptive immunity. The innate immune response is a non-specific response, meaning that it responds to pathogens in a generic way. It is the first-line defense since it can react immediately after infection. The adaptive immune response is effective after 4-5 days and shows exquisite specificity for its target antigens. Although the innate and adaptive immune responses are different in their mechanisms and action, synergy between them is essential for an intact, effective immune response (Chaplin, 2003).


**Innate immune response**

The first barriers that viruses come in contact with are mostly mucosal epithelial surfaces. These epithelial cell layers have tight cell-cell contacts and form a strong mechanical barrier. Other barriers can be present to prevent the viruses to interact with host target cells like low pH, host own microflora, digestive enzymes and a mucus layer (Tosi, 2005). Epithelial cells can also produce chemical substances that can have antiviral effects, like β-defensins (Quiñones-Mateu et al, 2003). Once the viruses attach to, invade and replicate in the host, they can immediately be recognized by different cells. This recognition and discrimination between self and viral structures is important and relies on germline-encoded ‘patterns recognition receptors’ (PRRs), which recognize patterns of repeated structural motifs. Examples of PRRs are macrophage mannose receptor, scavenger receptors and toll-like receptors (Trujillo et al, 2007; Koyama et al, 2008). These receptors can be present on the cell-surface, in endosomes or the cytoplasm of dendritic cells and macrophages, but also lymphocytes, neutrophils, eosinophils, epithelial cells and keratinocytes.

The pathogen-associated molecular patterns (PAMPs) that are recognized by these receptors during viral infections are nucleic acids, such as double and single stranded viral RNA and viral DNA, and viral proteins. Recognition can occur during early and later steps of the viral replication cycle or in a replication-independent way like phagocytosis of viral product (Finberg et al, 2007; Saito and Gale, 2007; Thompson and Iwasaki, 2008). Recognition of a PAMP starts an intracellular signaling cascade which ultimately promotes the expression of (proinflammatory) cytokines and chemokines. These molecules can cause an influx of other white blood cells and plasma proteins to the site of infection with diverse effects.

(i) The attracted **neutrophils** can recognize and destroy pathogens.

(ii) The attracted **monocytes** and immature **dendritic cells** can differentiate into tissue macrophages and mature dendritic cells respectively. These cells contribute to the generation of adaptive immunity.

(iii) Important attracted plasma proteins are the ones belonging to the **complement system**. Activation of the complement cascade leads to its main effector functions: recruitment of inflammatory cells, killing of pathogens, neutralization (which inhibits infection of susceptible cells) and
opsonization (which promotes phagocytosis). Cell-free virus and virus-infected cells can activate the complement system in the absence of specific antibodies.

(iii) The attracted natural killer (NK) cells are an important defense against intracellular infections. Once activated, they can recognize and destroy virus-infected cells and produce high levels of certain chemokines and cytokines. An important cytokine is interferon (IFN) \( \gamma \) (type II interferon) (Biron et al, 1999).

The type I interferons (IFN\( \alpha \) and IFN\( \beta \)) are important cytokines produced during the innate immunity. They activate the expression of more than 300 proteins in infected and neighboring uninfected cells, with antiviral, antiproliferative and immunomodulatory effects. The type I interferons are together with IFN \( \gamma \) responsible for different important effects during viral infection (Stark et al, 1998; Samuel, 2001; Schroder, 2004; Randall and Goodbourn, 2008).

(i) Induction of resistance to viral replication. Inhibition of the viral replication can occur in any step of the replication cycle. For example, degradation of cellular and viral RNA can be induced or transport of nucleocapsid into the nucleus can be prevented.

(ii) Upregulation of major histocompatibility complex class I (MHC-I) molecules and MHC class II (MHC-II) molecules expression and antigen presentation in all cells and induction of the expression of co-stimulatory molecules on macrophages and dendritic cells.

(iii) Activation of dendritic cells which induce their maturation in mature dendritic cells, highly effective antigen-presenting cells (APCs), and their migration to local lymphoid tissue. This is an important step for the induction of adaptive immunity.

Other components of the innate immunity are the innate-like lymphocytes (ILLs). These lymphocyte subsets express only a very limited diversity of receptors and do not need to undergo clonal expansion before responding to pathogens. One type of ILLs are the epithelial \( \gamma:\delta \) T cells. Little is known about which molecules stimulate the majority of these cells. These molecules appear to be expressed upon stress or transformation of epithelial cells. Activation of these cells can result in rapid production of cytokines and lysis of infected cells (Komori et al, 2006). Another
type of ILLs are the B-1 cells, a subset of the B cells. These cells are mostly present in the pleural and peritoneal cavities. Upon stimulation in the absence of antigens, these cells can produce ‘natural antibodies’. Natural antibodies are capable of neutralizing virus, directly or together with complement component (Jayasekera et al, 2007; Matter and Ochsenbein, 2008). A third type of ILLs are the NK T cells. Activation of NK T cells leads to release of cytokines, including IFNγ and interleukin (IL) 4, which then effect innate immunity and can lead to reduction in virus titers (Van Dommelen and Degli-Espostie, 2004; Ho et al, 2008).

**Adaptive immune response**

When the innate host defenses are bypassed, evaded or overwhelmed, an adaptive immune response is required. Dendritic cells, activated during the innate immune response, play a crucial role. These mature dendritic cells are very potent antigen presenting cells (APC), next to macrophages and B cells. The mature dendritic cell migrate to local lymph nodes, express co-stimulatory molecules and adhesion molecules and secrete chemokines that specifically attracts naïve T cells. These APC can present viral peptides on the cell-surface glycoproteins MHC-I and MHC-II after intracellular viral replication or ingestion of viral proteins. In the lymphoid tissue, the circulating naive T-lymphocytes can recognize these viral peptide:MHC complexes. This results in activation and proliferation (clonal expansion); followed by differentiation of the T-lymphocytes into armed effector T-lymphocytes or memory T-lymphocytes. IL2 production and expression of its receptor is crucial for the proliferation and differentiation in effector cells. These effector T-lymphocytes can respond to their target without co-stimulation and they express more cell-adhesion molecules which allow them to enter sites of infection (Chaplin, 2003).

Naive CD8⁺ T-lymphocytes differentiate into cytotoxic CD8⁺ effector T-lymphocytes, also called cytotoxic T lymphocytes (CTLs) with different effector functions (Harty et al, 2000; Wong and Pamer, 2003).

(i) Induction of apoptosis in virus-infected cells that display viral peptide fragments presented on MHC-I molecules (illustrated in Figure 1.4 (3)). Apoptosis is induced by releasing perforins, granzymes and granulysin.

(ii) Production of IFNγ which inhibits viral replication, induces increased expression of MHC-I proteins in infected cells and activates macrophages.
(iii) Production of tumor necrosis factor (TNF) α and β which can synergize with IFNγ in macrophage activation.

Naïve CD4⁺ T-lymphocytes differentiate into immature effector cells (Th0 cells) which differentiate further in either T helper 1 (Th1) cells or T helper 2 (Th2) cells. The factors that determine if Th1 or Th2 cells are generated are not fully understood. The cytokines produced by cells of the innate immune system in response to the virus play an important role. Th0 cells activated in the presence of IL4, especially when IL6 is also present, tend to differentiate into Th2 cells. CD4⁺ T-lymphocytes initially stimulated in the presence of IL12 and IFNγ tend to develop into Th1 cells (Santana and Rosenstein, 2003). Since during early virus infection IL12 (produced by dendritic cells and macrophages) and IFNγ (produced by NK cells and CD8⁺ cells) are present, Th1 cells are mostly dominating the viral CD4⁺ T-lymphocyte response. The consequences of inducing Th1 versus Th2 cells are important since both cell types lead to a different type of immune response (Zhu and Paul, 2008).

The effector functions of Th1 cells lead to a cell-mediated immune response.

(i) Activation of macrophages to potent antimicrobial effector cells by inducing the production of nitric oxide (NO) and superoxide ions (O₂).

(ii) Production of IL2 to induce T-lymphocyte proliferation and increasing numbers of effector cells.

(iii) Production of IFNγ which activates B-lymphocytes to produce opsonizing antibodies and up-regulates MHC-I molecules on cells promoting CD8⁺ T-lymphocyte function.

(iv) Stimulation of the production of new phagocytic cells in the bone marrow via hematopoietic growth factors IL3 and granulocyte macrophage colony-stimulating factor (GM-CSF).

(v) Recruitment of phagocytic cells to the site of infection by production of TNFα and TNFβ which activate endothelium to induce macrophage binding and extravasation and also by production of chemotactic factors.

The effector functions of Th2 cells provide a humoral immune response.

(i) Recognition of the peptide:MHC-II complex on the naïve B-lymphocyte activates the B-lymphocyte. Activation leads to proliferation of the
lymphocyte into antibody-secreting plasma cells or memory B-lymphocytes.

(ii) Production of cytokines like IL4, transforming growth factor (TGF) β, IL5 which influence the switching of different isotypes of the antibodies.

(iii) Production of IL10 which can inactivate macrophages.

More recently, two other CD4⁺ T-lymphocyte subsets have been described: the Th17 and the induced regulatory T (iTreg) cells. Th17 cells play a role in immune responses against extracellular bacteria, fungi and in autoimmunity. iTreg cells play a role in immune tolerance, lymphocyte homeostasis and regulation of immune responses (Zhu and Paul, 2008).

Figure 1.4: Recognition of a virus-infected cell followed by activation and lysis via three mechanisms: (1) via antibody-dependent, complement-mediated lysis (ADCML), (2) via antibody-dependent, cell-mediated cytotoxicity (ADCC) and (3) via cytotoxic T-lymphocytes (CTLs).
Naïve B-lymphocytes that are stimulated by Th2 cells, or directly by the pathogen, differentiate into plasma cells and secrete antibodies. These antibodies produced by the plasma cells have various ways to help overcome the infection and are found throughout the body. They can have a direct effect on free viruses (Hirsch 1982; Burton, 2002). Binding of antibodies to viruses can neutralize the virus by for example, blocking virus binding to surface receptors or interfering with the fusion of the virus membrane. It can also opsonize the viruses, in the presence or absence of the complement, to promote up-take and removal by phagocytes via Fc or complement receptor. Finally, viruses can be destroyed (virolysis) via antibodies and complement activation. Antibodies can also have an effect on virus-infected cells. Antibodies can bind to viral proteins that are incorporated into the plasma membrane of the infected cell during viral replication. This is seen with enveloped viruses and can induce lysis of the infected cells through two mechanisms (Sissons and Oldstone, 1980). One mechanism is the antibody-dependent, cell-mediated cytotoxicity (ADCC). Antibodies bound to the surface of the infected cell can activate phagocytic cells through their Fc receptor which can result in lysis of the cell (illustrated in Figure 1.4 (2)). Cells that can induce ADCC are monocytes, macrophages, neutrophils and natural killer cells (Koren, 1983). The other mechanism is antibody-dependent, complement-mediated lysis (ADCML). Antibodies bound to the surface of the infected cell can lead to binding and activation of the complement system which can lead to lysis of the cell (illustrated in Figure 1.4 (1)).

**Immune evasion**

The host has developed a complex variety of protective mechanisms to control and eliminate pathogens like viruses. On the other hand, viruses have developed an array of immune evasion mechanisms that counteract this and allow the viruses to replicate and spread in the host. Generally, each virus uses multiple strategies for immune evasion. The larger the viral genome, the more diverse mechanisms may be utilized (Vossen et al, 2002). Overall, different viral immune evasion mechanisms have been detected on different levels of the host innate and adaptive immune response.
Viral proteins can bind to cytokines and interferons, inhibit cytokine synthesis, oppose apoptosis or interfere with different signaling pathways emanating from the patterns recognition receptors (Schröder and Bowie, 2007). The adaptive immune response mechanisms can also be evaded in different ways. These immune evasion strategies can be focused on interfering with the antigen presentation via the MHC molecules, down-regulating the expression of co-stimulating molecules, evading NK cell responses, evasion from cytotoxic T lymphocytes or neutralizing antibodies by antigenic variation, latency, targeting immune cells, interfering with apoptosis of the virus-infected host cells, targeting cytokines and chemokines or interfering with the complement system (reviewed by Vossen et al, 2002; Favoreel et al, 2003; Iannello et al, 2006).

For example, different immune evasion mechanisms have been described for mouse hepatitis virus (MHV), a group 2a coronavirus. The N protein of MHV can act as a type I interferon antagonist while the S protein has an Fc receptor activity which could protect the infected cell from antibody-mediated lysis (Oleszak, 1993; Ye et al, 2007). Fc receptor activity has also been described for other coronaviruses, namely bovine coronavirus (BCV), infectious bronchitis virus (IBV) and transmissible gastroenteritis virus (TGEV) (Oleszak et al, 1993, 1995). Also, decreased expression of MHC-I has been described during chronic MHV infection which can impair the lysis by the cytotoxic T cells (Redwine et al, 2001). MHV can also impair the production of NK cell by infection and subsequent apoptosis of these cells (Lehoux et al, 2004). Finally, cytotoxic T cell escape variants of MHV with mutations in a single epitope from the spike glycoprotein could play a role in escaping the immune system during chronic infections (Butler et al, 2008).

Three (possible) immune evasion processes have been described for FCoV: formation of quasispecies during chronic FECV infection, the antibody-dependent enhancement of infectivity (ADEI) and a FIPV induced T cell depletion. These mechanisms will be discussed in the next part (1.2.2).
1.2.2 FCoV

The immune response against infections is a complicated system with different effectors and their functions. For FCoV, parts of the immune response have been studied, but still a lot has to be clarified. It becomes clear that the outcome of a FCoV infection is determined by a complicated interaction of the virus with the immune system of the cat.

**FECV**

Which immune mechanism is responsible for overcoming an FECV infection has not yet been clarified. Since FECV replication is largely confined to the gut, the local gut immunity is probably important to fight the infection. Maternal antibodies (immunoglobulin (Ig) G antibodies in the colostrum and the IgA antibodies in the milk) are protective in kittens (Pedersen, 1987). Therefore, most kittens are infected at the age of 9-10 weeks, when the maternal immunity wanes (Foley et al, 1997b; Pedersen et al, 2008). Earlier infections from 2 weeks onwards are possible (Harpold et al, 1999; Gut et al, 1999). In adult cats, a systemic immune response is present as shown by the presence of antibodies 3 to 4 weeks after infection, activation of monocytes/macrophages and the generalized lymphatic B and T cell hyperplasia (Haagmans et al, 1996; Meli et al, 2004; Kipar et al, 2001, 2006b). Also FCoV containing immune-complexes have been demonstrated in the plasma of healthy FCoV infected cats (Kipar et al, 1999). Containing an FECV infection has been suggested to be associated with high levels of IL10 which could avoid excessive macrophage activation (Kipar et al, 2006b). Also an antibody response against the S protein has been suggested to contribute to the clearance of the infection (Gonon et al, 1999).

Although the immunity generated during an FECV infection is not fully understood, several characteristics are known. The immunity seems to lack memory, since recovered cats can be infected with the same or different strains of the virus (Addie et al, 2003; Pedersen et al, 2008). The immunity is variable in strength, since some cats overcome the infection while others show persistent or intermittent/recurrent infection. How FECV avoids clearance in persistently infected cats is not fully understood but it could be due to increasing number of mutations in time in the S protein.
protein and formation of quasispecies (Herrewegh et al, 1997). Viral quasispecies have closely related, yet nonidentical genomes due to mutations. As a result from these mutations, the virus may escape from the immune system leading to a chronic carrier state.

**FIPV**

It is generally thought that the cell-mediated immunity plays a decisive role in partial or full control of FIP. Following observations and strong indications support this hypothesis.

(i) More severe intestinal lesions and peritonitis due to FIPV were seen in cats lacking T-lymphocytes after thymectomy (Hayashi et al, 1983).

(ii) Cats showing a stronger delayed type of hypersensibility reaction against FIPV, which is a cell-mediated reaction, had a longer surviving time (Weiss and Cox, 1989).

(iii) Higher incidence of FIP is seen if concurrent infection is present with feline immunodeficiency virus, which suppresses the cell-mediated immunity (Poland et al, 1996).

(iv) In FIP survivors a Th1 type T-cell response has been seen, shown by the detection of intracellular TNFα (de Groot-Mijnes et al, 2004).

(v) No difference is seen in the antibody concentration and fluctuations between survivors and non-survivors of FIP (de Groot-Mijnes et al, 2005).

A strong cell-mediated immune response seems to be protective against FIP. The S protein appears to be the main CD8⁺ and CD4⁺ T-lymphocyte antigen (de Groot-Mijnes et al, 2005). In cases where FIPV is not eliminated, T- and B-lymphocyte apoptosis and depletion is observed in lymphatic and peripheral tissues (Haagmans et al, 1996; Kipar et al, 2001). The T-lymphocyte depletion (CD8⁺ and CD4⁺) occurs early in infection and correlates with enhanced viral replication (de Groot-Mijnes et al, 2005). This depletion is induced indirectly since no direct effect of virions or viral proteins has been detected (Haagmans et al, 1996). The efficacy of early T-lymphocyte responses most likely determines to what extent the initial wave of infection can be contained and would be the decisive factor in disease progression. If the first response is too weak, following phases of enhanced viral replication will
further reduce T-lymphocyte numbers and ultimately resulting in total loss of immune control and unchecked viral replication (de Groot-Mijnes et al, 2005).

Immunity against FCoV seems to be associated with an imbalance in TNFα and IFNγ. In cats with FIP, higher systemic levels of TNFα and lower levels of IFNγ were detected (Kiss et al, 2004; Gelain et al, 2006). TNFα could play a role in the lymphocyte apoptosis, while the lower levels of IFNγ, together with the detected lower levels of IL12, can contribute to the impaired cellular immune response during infection (Gelain et al, 2006; Kipar et al, 2006b).

The humoral immunity is activated during the development of FIP, but is not protective and even contributes to the pathogenesis of FIP. Virus-neutralizing antibodies are present and B-lymphocytes and plasma-cells producing coronavirus-specific antibodies are detected in the lesions (Kipar et al, 1998b; de Groot-Mijnes et al, 2005). An increase of plasma cells in the peripheral blood is also seen (Takano et al, 2009). This B-lymphocyte activation and maturation seem to be induced by the presence of stimulating factors like IL1, IL6 and B-cell activating factor (Hasegawa and Hasegawa, 1991; Goitsuka et al, 1990, 1991; Takano et al, 2009). This activation of the humoral immunity is not protecting the cat against FIP. Antibodies seem to have a close involvement in the FIP pathogenesis. They seem to play an important role in the maintenance of the inflammatory process in FIP (Kipar et al, 1998b). Immune-complex deposition and complement activation could also contribute further to the pathogenesis (Jacobse-Geels et al, 1980, 1982). One immune evasion mechanism has been described via which cell free FIPV can evade antibody-dependent lysis, namely the antibody-dependent enhancement of infectivity (ADEI). ADEI is seen in vitro, were antibodies against epitopes on the S or M protein can apparently opsonize the virus and enhance the infection of monocytes/macrophages via Fc receptor mediated internalization (Hohdatsu et al, 1991; Corapi et al, 1992; Olsen et al, 1992, 1993; Olsen and Scott, 1993; Corapi et al, 1995; Takano et al, 2008). It is also seen as the reason why a more fulminating disease progression of FIP is seen in experimentally seropositive animals after inoculation (Pedersen and Boyle, 1980; Weiss and Scott, 1981a,b; Pedersen and Black, 1983; Vennema et al, 1990). In natural infections of seropositive animals no enhancement of infectivity seems present. Epidemiological data suggest that seropositive cats develop a protective immunity against natural infection rather than
an increased sensitivity. Seropositive cats showed a normal FIP progression and did not die more rapidly nor did the disease seem to be more severe (Addie et al, 1995).

The immune system of the cat seems to play an important role in the outcome of an FCoV infection. Figure 5.1 gives an overview of the different outcomes of an FCoV infection and the known involvement of parts of the immune response. There are still questions that need to be answered. Which virological and/or immunological factors lead to the carrier state? Which (genetic) factor is responsible for the resistance to FCoV infection? Why is the humoral immune response not protective? These and many more issues in FCoV research have to be resolved, which hopefully leads to an effective diagnosis, treatment and/or prevention of FIP in the future.
Introduction

Figure 1.5: The different outcomes of an infection with the two biotypes of FCoV, namely FECV and FIPV and the involvement of elements of the immune response.
Chapter 1

References


virus antigen and the interrelationship of these viral infections in free-ranging lions in east Africa. Clin Diagn Lab Immunol 3, 554-562


shows cell tropism in neutralizing activity after viral absorption into the cells. Arch Virol 145, 1-12


Zook B, King N, Robinson R, McCombs H (1968) Ultrastructural evidence for the viral etiology of feline infectious peritonitis. Pathol Vet 5, 91-95

Aims
Aims of the thesis

FECV is an avirulent variant of the feline coronaviruses and is endemic in the cat population. Occasionally, it can mutate to FIPV, the virulent variant. FIP is for veterinarians and cat owners a difficult and, in most cases, a hopeless situation because (i) FIP is difficult to diagnose, (ii) there is no effective treatment and (iii) there is no effective prevention strategy. The pathogenesis of FIP and the relationship between the different FCoV biotypes has been partly clarified. Still, more aspects of the pathogenesis have to be elucidated, especially the interaction between the viruses (and the virus-infected cell) with the immune system. This can lead to a more rational design of treatment or prevention strategies.

The infected blood monocytes play an important role in the pathogenesis of FIPV. These cells disseminate the virus and start the induction of the lesions (granulomas). In a FIP cat, infected monocytes/macrophages are found in the granulomas, the exudates and the blood. In one way or another, these infected cells succeed in staying alive and transmitting virus to new, susceptible cells in the presence of high antibody concentrations. Infected cells are normally eliminated by the adaptive immune system through antibody-mediated lysis or cell-mediated lysis. For the antibody-mediated lysis, the presence of viral antigens on the surface of infected cells is important for the recognition of these cells by antibodies and the subsequent destruction by the immune system.

For FIPV the infected monocytes/macrophages are poorly studied and their interactions with effectors of the immune system are unknown. Therefore, the major aim of this thesis was to determine the virus-target cell interaction and search for possible immune evasion processes.

First, the replication characteristics (virus production and antigen expression) of FIPV and FECV in the feline blood monocytes were examined. This allowed to determine whether an *in vitro* difference between the replication of FIPV and FECV is present which correlate with the different pathogenesis of these viruses *in vivo* (Chapter 3). Secondly, the FIPV-infected cells present in cat with clinical FIP were
examined \textit{ex vivo}. The presence of two immunologic targets, viral antigens and MHC-I, on their surface was determined (Chapter 4). In Chapter 5 it was examined whether FIPV-infected monocytes escape from antibody-dependent complement mediated lysis, an important immunological defense mechanism against viruses.
Chapter 3

Replication of feline coronaviruses in feline peripheral blood monocytes

Adapted from:
Summary

Feline infectious peritonitis virus (FIPV) causes the most lethal viral infection in cats: FIP. The closely related feline enteric coronavirus (FECV) generally causes mild enteritis. Why these feline coronaviruses manifest so differently in vivo is not known. In this study, infection kinetics (titers and antigen expression) of FIPV 79-1146, and FECV 79-1683, were determined in peripheral blood monocytes from 3 donor cats and compared to those in Crandell feline kidney (CrFK) cells. The infection kinetics in monocytes were host dependent. Monocytes from 1 cat were resistant to both FIPV- and FECV-infection. Monocytes from the other 2 cats could initially be infected by both FIPV and FECV but FIPV-infection was sustained in monocytes of only one cat. FECV-infection was never sustained and viral production was up to 100 times lower than in FIPV-infected monocytes. In CrFK cells, FIPV and FECV infection kinetics did not differ. In monocytes of a larger cat population (n=19) the 3 infection patterns were also found. Considering all 22 investigated cats, 3/22 were not susceptible for FIPV and FECV. The rest could be infected with FECV and FIPV but 10/22 cats had monocytes that only sustained FIPV infection and 9/22 sustained neither FIPV nor FECV infection.
Introduction

Two coronaviruses are described in cats: feline infectious peritonitis virus (FIPV) and feline enteric coronavirus (FECV). These feline coronaviruses are spread worldwide and infect both domestic cats as non-domestic cats of the Felidae family. An infection with FECV is usually subclinical, except in young kittens where it may cause mild to severe diarrhea (Pedersen et al, 1981; Kipar et al, 1998). In contrast, FIPV infection causes a chronic and very often fatal pleuritis/peritonitis. It is the most important cause of death of infectious origin in cats. Two forms of FIP exist: the effusive or wet form with the typical effusions in body cavities and the less common non-effusive or dry form (Pedersen, 1983). Characteristic lesions of both forms are granulomas on the surface of target tissues. Despite the large biological differences, more than 98% of the genome is identical in FIPV and FECV isolates from the same environment (Vennema et al, 1995). Therefore, it has been proposed that FIPV arises from FECV by mutation but the exact mutation and the inducing factors have not yet been clarified (Poland et al, 1996; Vennema et al, 1998).

The main difference between FECV and FIPV is the invasive nature of FIPV. FECV replicates mainly locally, in enterocytes of the intestine, whereas FIPV also infects blood monocytes and spreads systemically (Weiss and Scott, 1981a, b). The reason for this pathogenic difference is not understood. After infiltration of infected monocytes in the perivascular tissue, the infected monocytes and surrounding cells release numerous chemotactic and vasoactive factors (Weiss et al, 1988; Goitsuka et al, 1990, 1991). This leads to vasodilatation and increased vascular permeability and attraction of new monocytes to the area, which can be infected in turn. The outcome of the inflammatory reaction is a characteristic vasculitis which causes the venules to leak large amounts of protein rich plasma into the body cavity. The release of progeny virus also leads to the formation of virus-antibody-complement complexes which are concentrated around the small venules in the target organs (Jacobse-Geels et al, 1980). These complexes further activate inflammation.

Although the difference between FIPV and FECV is very clear in vivo, it is not in vitro. The first in vitro characterization of FIPV strain 79-1146 and FECV strain 79-1683 was done by McKeirnan et al (1987) in Crandell feline kidney (CrFK) cells. They found similar growth curves for FIPV and FECV. The replication of FIPV and
FECV was also studied in peritoneal macrophages (Stoddart and Scott, 1989). It was reported that FECV infected fewer macrophages and reached lower production titers than FIPV. The \textit{in vivo} relevance of these infection studies is most likely higher than those performed in a continuous cell line. But, until now, the FIPV and FECV replication cycles have never been studied in the \textit{in vivo} target/carrier cell of FIPV: the feline blood monocyte.

In the present study, we present the \textit{in vitro} replication kinetics of FIPV and FECV in the target cell of FIPV, the blood monocyte. It was found that the replication kinetics were dependent on the origin of the cells. No differences between FIPV and FECV were found in CrFK cells.
Materials and Methods

Viruses. FIPV strain 79-1146 and FECV strain 79-1683, passaged on CrFK cells, were used (McKiernan et al, 1981). FECV strain 79-1683 was obtained from the American Type Culture Collection (ATCC) and FIPV strain 79-1146 was kindly provided by Dr. Rottier and Dr. Egberink (Utrecht University, the Netherlands).

Antibodies. Polyclonal antibodies originating from cats infected with FIPV 79-1146 were kindly provided by Dr. Rottier and Dr. Egberink (Utrecht University, the Netherlands). These antibodies were purified and biotinylated according to manufacturer’s instructions (Amersham Bioscience, Buckinghamshire, UK). The monoclonal antibodies 7-4-1, F19-1, E22-2, recognizing respectively the S-, M- and N-protein, were kindly provided by Dr. Hohdatsu (Kitasato University, Japan). A monocyte marker, DH59B, recognizing CD 172a was purchased from Veterinary Medical Research and Development (Pullman, Washington, USA).

Cats. Three cats of a non-specific breed from a FCoV free closed household were used as blood donors for the extensive infection kinetics study. Seventeen stray cats brought to the clinic of small animals in the Faculty of Veterinary Medicine (Ghent University) and 2 SPF cats were used for a study on the distribution of the infection kinetics patterns. The sex and feline leukemia virus (FeLV), feline immunodeficiency virus (FIV) and feline coronavirus (FCoV) status of the cats are listed in Table 3.1.

Isolation of blood monocytes. Six ml blood was collected on heparin (15 U/ml) (Leo, Zaventem, Belgium) from the vena jugularis and blood mononuclear cells were separated on Ficoll-Paque (Pharmacia Biotech AB, Uppsala, Sweden) following manufacturer’s instructions. Mononuclear cells were resuspended in RPMI-1640 (Gibco BRL, Merelbeke, Belgium) medium containing 10% fetal bovine serum, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 10 U/ml heparin, 1mM sodium pyruvate and 1% non-essential aminoacids 100x (Gibco BRL). Afterwards, cells were seeded in a 24-well dish with cell culture coating (Nunc A/S, Roskilde, Denmark) at a concentration of
2×10^6 cells/ml and cultivated at 37°C with 5% CO₂. Non-adherent cells were removed by washing the dishes twice with RPMI-1640 at 2 and 24 hours after seeding. The adherent cells consisted for 86 ± 7% of monocytes (as assessed by fluorescent staining with the monocyte marker DH59B).

**Table 3.1: Sex and feline leukemia virus (FeLV), feline immuno-deficiency virus (FIV) and feline coronavirus (FCoV) status of the cats.**

<table>
<thead>
<tr>
<th>Cat n°</th>
<th>Sex</th>
<th>FeLV antigen</th>
<th>FIV antibody</th>
<th>FCoV titer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Closed household</td>
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<tr>
<td>1</td>
<td>M</td>
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<tr>
<td>Population of stray cat</td>
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<td>SPF cats</td>
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</table>

\( ^a \) M: male, F: female

\( ^b \) Tested on plasma samples with SNAP® FIV Antibody/FeLV Antigen Combo Test (IDEXX)

\( ^c \) IPMA antibody titer
**Inoculation of CrFK cells and monocytes.** CrFK cells and monocytes were inoculated with FIPV strain 79-1146 or FECV strain 79-1683 at a multiplicity of infection (moi) of 5. After 1 hour incubation at 37°C with 5% CO₂, cells were washed 3 times with RPMI-1640 and further incubated in medium.

**Growth curves of FCoV.** At different time points post inoculation, culture medium was harvested and centrifuged at 400×g for 10 minutes. The supernatants were used for determination of extracellular virus titers. The cells were removed from the well by scraping and added to the pellet for determination of intracellular virus titer. Virus was released from the cells by 2 freeze-thaw cycles. The samples were stored at −70°C until titration. Both intra- and extracellular virus titers were assessed by a 50% tissue culture infective dose (TCID₅₀) assay using CrFK cells. The fifty percent end-point was calculated according to the method of Reed and Muench (1938). A virus inactivation curve was determined by keeping cell free virus in medium at 37°C with 5% CO₂. Samples were taken at different time points and stored at -70°C until titration. Three independent assays were carried out and the inactivation curve was calculated by linear regression.

**Visualization of viral antigens in FCoV infected cells.** At different time points post inoculation, cells seeded on glass coverslips, were fixed with 1% formaldehyde. Surface-expressed viral proteins were labeled with biotinylated anti-FIPV polyclonal cat antibodies and streptavidin-FITC (Molecular Probes, Eugene, Oregon, USA). After permeabilization with 0.1% Triton X-100 (Sigma-Aldrich GmbH, Steinheim, Germany), cytoplasmic viral proteins were stained with a mixture of monoclonal antibodies (7-4-1, F19- 1 and E22-2) and with goat anti-mouse-Texas Red (Molecular Probes). Finally, the glass coverslips were mounted on microscope slides using glycerin-PBS solution (0.9:0.1, vol/vol) with 2.5% 1,4-diazabicyclo(2,2,2)octane (Janssen Chimica, Beerse, Belgium) and analyzed by fluorescence microscopy. For the stray cats and SPF cats, only cytoplasmic viral proteins were stained with FITC labeled anti-FIPV antibodies (VMRD Inc, Pullman, Washington, USA).
Confocal laser scanning microscopy. The samples were stained to visualize the cytoplasmic and the surface-expressed viral proteins as described above and examined with a Leica TCS SP2 laser scanning spectral confocal system (Leica Microsystems GmbH, Wetzlar, Germany) linked to a DM IRB inverted microscope (Leica Microsystems). Argon and Helium/Neon laser lights were used to excite FITC (488 nm line) and Texas-Red (543 nm line) fluorochromes. The images were obtained and processed with Leica confocal software.

Statistical analysis. All experiments were repeated twice or more. The “area under the curve” was calculated for each experiment. Triplicate assays were compared using a Mann-Whitney U test. Statistical analyses were performed with SPSS 11.0 (SPSS Inc. Chicago, Illinois, USA).
Results

Growth curves of feline coronaviruses in CrFK cells. The growth curves of FIPV and FECV in CrFK cells are given in Figure 3.1. Production of progeny virus started between 3 and 6 hours post inoculation (hpi) and increased strongly until 12 hpi. Between 12 and 24 hpi there was only a slight increase of virus titers to reach a maximum of $6.8 \log_{10} TCID_{50}/10^6$ cells at 24 hpi. There was no significant difference between the growth curves of FIPV and FECV.

Figure 3.1: Kinetics of FIPV and FECV replication in CrFK cells. At designated time points post inoculation, the intracellular (●) and extracellular (△) virus titres were determined. The dashed line represents the inactivation curve and the dotted line is the detection limit. The data represent means ± SD of triplicate assays.

Expression kinetics of cytoplasmic and surface-expressed viral antigens in feline coronavirus-infected CrFK cells. Figure 3.2 shows that the first viral antigen positive cells appeared between 3 and 6 hpi. Between 12 and 24 hpi, there was a vast increase of infected cells. At 24 hpi, 86% of the cells showed cytoplasmic expression of viral proteins and 75% surface expression. There is no significant difference (area under the curve) between the FIPV curve and the FECV curve. The amount of infectious virus produced per cell can, theoretically, be calculated from
the virus titers and the percentage of infected cells. For both FIPV- and FECV-infected CrFK cells, productivity was less than 10 infectious viruses per infected cell.

Figure 3.2: Kinetics of expression of viral antigens in FIPV and FECV infected CrFK cells. At designated time points post inoculation, the cells were fixed and cytoplasmic (▲) and surface-expressed (●) viral proteins were visualized with an immunofluorescence staining. The data represent means ± SD of triplicate assays.

Growth curves of feline coronaviruses in monocytes. The growth curves of FIPV and FECV in monocytes varied between the three donor cats from a closed household. Figures 3.3a and 3.4a show that the production of FIPV started between 3 and 6 hpi for both cat 1 and 2. Between 12 and 24 hpi there was a slight increase in virus titer for cat 1 whereas the curve from cat 2 reached a plateau at 12 hpi. The virus release curves were similar (Figure 3.3c and 3.4c). The growth curves of cat 1 for FECV showed a low-level production (Figure 3.3b and d). The growth curves of cat 2 for FECV began with a slight titer increase, similar to the FIPV growth curve, but then the virus titer decreased with a slope comparable to the inactivation curve (Figure 3.4b and d). These findings suggest that monocytes could be infected by FECV but that the cells did not sustain a productive infection. Figure 3.5 shows that the growth curves for cat 3 followed the inactivation curve, suggesting that there was no progeny virus produced.
Replication of FCoV in monocytes

Figure 3.1: Kinetics of FIPV and FECV replication in blood monocytes from cat 1.

Virus titers cat 1

- mean titre, — inactivation curve, — detection limit

FIPV

Virus titer (log_{10} TCID_{50}/10^6 cells)

Intracellular titer

Time post inoculation (h)

0 3 6 9 12 15 18 21 24

Extracellular titer

Virus titer (log_{10} TCID_{50}/10^6 cells)

Time post inoculation (h)

0 3 6 9 12 15 18 21 24

FECV

Viral antigen expression cat 1

- exp. 1, — exp. 2, — exp. 3

FIPV

Cytoplasmic expression

Percentage of positive cells

Time post inoculation (h)

0 6 12 18 24

Surface expression

Percentage of positive cells

Time post inoculation (h)

0 6 12 18 24

FECV

Percentage of positive cells

Time post inoculation (h)

0 6 12 18 24
Figure 3.4: Kinetics of FIPV and FECV replication in blood mononcytes from cat 2.
Figure 3.5: Kinetics of FIPV and FECV replication in blood monocytes from cat 3.
Expression kinetics of cytoplasmic and surface-expressed viral antigens in feline coronavirus-infected monocytes. Figure 3.6 shows confocal images of cytoplasmic and surface-expressed viral antigens in monocytes infected with FIPV 79-1146. Surface expression was only detected in an average of 49% of the infected monocytes (24 hpi). No differences in the amount of infected cells with surface expression were seen between the cats or between FIPV and FECV infection. Depending on the cell, the amount of viral antigens expressed on the surface varied. The majority of the infected monocytes showed a small amount of surface-expressed viral proteins (Figure 3.6, lane 1). Some showed a larger amount of surface-expressed viral proteins (Figure 3.6, lane 2).

The antigen expression kinetics varied between the donor cats. Figure 3.3e and f show the FIPV and FECV cytoplasmic expression kinetics for cat 1. The percentage of FIPV infected cells with cytoplasmic expression increased till 24 hpi. The infection of monocytes with FECV initiated in the same manner but at 12 hpi the curve started to decline. The cytoplasmic expression in monocytes of cat 2 is shown in Figure 3.4e and f. Infection with FIPV or FECV led to the same expression kinetics. After an increase until 6 or 12 hpi the percentage of cells with viral expression decreased rapidly. The number of FECV infected monocytes was lower than the FIPV-infected monocytes. The FIPV and FECV surface expression, for both cat 1 and 2, followed the same curve as the cytoplasmic expression but at a lower percentage (Figure 3.3g and h; Figure 3.4g and h). The results of cat 3 were quite different from cat 1 and 2. Here, viral antigen positive monocytes were not found.

Knowing the total production of infectious progeny virus and the number of infected cells, it can be calculated that FIPV-infected monocytes from both cat 1 and 2 have produced approximately 200 infectious viruses per infected cell at 12 h post inoculation. FECV-infected monocytes from cat 1 produced 10 times less progeny virus at 12 h post inoculation whereas the FECV-infected monocytes from cat 2 produced the same amount of progeny virus as the FIPV-infected monocytes.
Figure 3.6: Internal expression and surface expression of viral proteins in two monocytes (1 and 2) infected with FIPV 79-1146, visualized by confocal microscopy. Bar=5µm.

**Infection kinetics in a larger population of cats.** In order to clarify the prevalence of the patterns of viral replication observed in this study in a bigger cat population, the antigen expression kinetics were studied in 17 stray cats and 2 SPF cats for both FIPV and FECV. The antigen expression was visualized at 0, 12 and 24 hpi. The results are presented in Figure 3.7. The 3 different expression kinetics that were found in monocytes from the closed household cats were also seen in monocytes from the stray cats and the SPF cats. Within this population of 19 cats, the monocytes isolated from 9 cats showed a continuous increase in viral antigen positive cells during a 24 hour time span after inoculation with FIPV. When these monocytes were inoculated with FECV, the number of viral antigen positive cells increased until 12 hpi and then diminished. In monocytes from 8 cats, the percentages of both FIPV- and FECV-infected cells increased until 12 hpi and then decreased. The monocytes from 2 cats were resistant to infection.
Figure 3.7: Kinetics of FIPV and FECSV replication in blood monocytes from 17 stray cats (solid line) and 2 SPF cats (dashed line). Each curve represents the FIPV and FECSV infection kinetics from 1 cat.
Replication of FCoV in monocytes

Discussion

In this study, \textit{in vitro} infection kinetics of FIPV (strain 79-1146) and FECV (strain 79-1683) were established in peripheral blood monocytes from 22 cats (3 cats of a closed household, 17 stray cats and 2 SPF cats). It is the first time that infection studies were performed in peripheral blood monocytes, the host/carrier cell of FIPV. Three distinct patterns were found in the infection studies.

Monocytes from 3 cats were not infected by either strain (first pattern). The reason for the insusceptibility of these cells is not yet clear. Virus particles were detected in the cells shortly after inoculation of the cells but no production of viral antigens was observed using polyclonal antibodies (data not shown). Thus, it seems that no new viral proteins were formed. This suggests that the block of infection is located after entry of the virus but before (or at) the translation step. \textit{In vivo}, resistance to FIPV infection has been observed in experimental inoculations. After inoculation with a lethal dose of FIPV, a varying part of the cats (depending on experiment 8-50\%) showed no clinical signs and some of them remained seronegative (Weiss and Cox, 1989; Poland et al, 1996). This was also seen in control groups of vaccination trials (no vaccination, only FIPV challenged) (McArdle et al, 1995; Scott et al, 1995).

Resistance to FCoV infection has also been suggested to occur in natural infections in the field (Addie and Jarrett, 2001). A small percentage of cats in FCoV endemic households had no shedding, remained seronegative or had a low antibody titer over a time period of 5 years. It would be most interesting to investigate the correlation between \textit{in vitro} and \textit{in vivo} resistance to FCoV. This might give perspectives for selection of cats insusceptible for FIP.

Monocytes from 10 cats showed an increase of FIPV antigen positive cells till 24 hpi whereas the amount of FECV antigen positive cells dropped after 12 hpi. This shows that the FIPV infection was sustained whereas the FECV infection was not sustained (second pattern). Monocytes from 9 cats did not sustain FIPV nor FECV infection since the number of viral antigen positive cells dropped after 6 or 12 hpi (third pattern). The drop in antigen positive cells after 6 or 12 hours post inoculation (hpi) may be explained by the fact that the infected cells died due to infection and were washed away during the staining. However, the same kinetics were found with staining in suspension, a technique which prevents cell loss (data not shown).
Another explanation is that monocytes stopped producing viral proteins and assembling new virions. The extracellular virus titers indeed showed that (almost) no new progeny virus was produced between 12 and 24 hpi. Some graphs show differences in virus titers between 2 experiments (with the same virus and with monocytes from the same donor cat) of up to 2 log_{10} units. These differences are intrinsic to working with primary cells and are reported in viral infection studies with porcine and equine monocytes as well (Nauwynck and Pensaert, 1994; van der Meulen et al, 2000).

Although FECV initially infects monocytes, the infection is never sustained. This implies that FECV might reach the blood circulation \textit{in vivo}. In several studies, healthy cats from FCoV endemic households were investigated (Egberink et al, 1995; Herrewegh et al, 1995, 1997; Gunn-Moore et al, 1998; Meli et al, 2004; Simons et al, 2005). In such households, where the FCoV was most likely FECV, a part of these healthy cats were viraemic for FCoV. FCoV was detected both in plasma and in monocytes. Therefore, it may be hypothesized that when FECV reaches the blood circulation, the lack of sustainability and long-term production of progeny virus (the total virus production was up to 100 times lower in FECV-infected monocytes) may be the reason for the lack of disease progress. This might form the basis for the difference with FIPV since FIPV infection is sustained and reaches higher titers. However, the non-sustained FECV infection might also be attributed to the virus strain that was used. Although FECV 79-1683 is a reference strain, it may act differently from other FECV strains due to its deletion in the 7b ORF (Vennema et al, 1992). It has been described that loss of the 7ab ORFs results in loss in virulence (Haijema et al, 2004). It could be that this loss in virulence is translated in loss of the ability to replicate efficiently in monocytes. Thus, whether the hampered replication of strain 79-1683 in monocytes is a universal property of FECV strains or only of 7b deleted/mutated strains, remains to be determined.

The different FIPV infection kinetics depending on the cat from which the monocytes were isolated suggests that cellular factors, influenced by genetic background and/or differentiation/activation status, are very important in determining the outcome of a FIPV infection. In an infection kinetics study where another cell type, feline peritoneal macrophages, was used, different results in the antigen expression kinetics were obtained (Stoddart and Scott, 1989). The number of
FECV infected peritoneal macrophages was lower than the number of FIPV infected peritoneal macrophages throughout the infection kinetics. Since the viral antigen kinetics was only performed until 14 hpi, a possible drop in antigen expression, as reported here, could not be evaluated. In contrast, our results suggest that FIPV and FECV can initially infect the same amount of cells but at 24 hpi, differences in sustainability of the infection are prominent. Since the same viruses were used as in our study, the different results are most probably due to cellular factors and/or a different differentiation status of the cells. Differences in susceptibility depending on the differentiation and/or activation status of the monocytes/macrophages has been reported for different viruses such as porcine reproductive and respiratory syndrome virus, caprine arthritis-encephalitis virus, suid herpes virus 1, herpes simplex virus, human immunodeficiency virus type 1 and Maedi-Visna virus (Duan et al, 1997; Nauwynck, 1993; Zink et al, 2002). The differences in activation status might explain the discrepancy between our results and those of Stoddart and Scott (1989).

What this variation in susceptibility and sustainability means for the pathogenesis of FECV and FIPV in vivo, remains to be elucidated. In an inoculation study using FIPV 79-1146, different patterns of disease progression were detected, based upon survival time: progressors (rapid, intermediate and delayed) and survivors (prolonged and long-term) (de Groot-Mijnes et al, 2005). With natural in vivo FCoV infection, different clinical outcomes (besides resistance to FCoV) have been described: persistent carrier, transiently infection and development of FIP (Addie et al, 2003). It is not clear what the viral and host factors are that determine the different clinical outcomes. Since in the inoculation study the same strain (FIPV-79-1146) was used and considering the fact that in the field cats are often infected with the same strain of FCoV, it is likely that genomic variation between cats contributes to a different clinical outcome. A genetic background was also suggested during a field study with pure-bred cats, in which it was shown that susceptibility to FIP is indeed inheritable (Foley and Pedersen, 1996). A possible explanation for the different disease progression is the possibility of the cats to develop an efficient T-cell response (de Groot-Mijnes et al, 2005). However, it could also be that the susceptibility of the monocytes to FIPV plays a role, considering the results presented here. It would be interesting to investigate if cats that show a different outcome to an experimental or natural infection also show different infection
kinetics in vitro. This might be important since a correlation between in vitro and in vivo infection kinetics would allow easy screening and selection.

In this study, it was shown that viral proteins can be expressed on the surface of FCoV infected cells. However, only a part of the infected cells showed surface-expressed viral antigens. On 24 hpi, 87% of the infected CrFK cells and 49% of the infected monocytes showed surface-expressed viral antigens. S and M proteins, but no N proteins were found on the cell surface of both CrFK cells and monocytes using specific monoclonal antibodies (data not shown). This indicates that the observed surface expression does not represent virus particles. Possible explanations for the observed differences in amount of surface-expressed viral antigens could be the retention of part of the viral proteins or spontaneous internalization of the surface-expressed viral antigens. Retention of viral proteins has been described for porcine coronavirus (Schwegmann-Wessels et al, 2004). Spontaneous internalization of viral proteins has been described for suid herpes virus 1 (Van Minnebruggen et al, 2004).

The presence of viral antigens on the cell surface can be of importance for the recognition and elimination of infected cells by the immune system. Binding of virus-specific antibodies to viral proteins present on the surface, makes infected cells recognizable for the classical complement pathway, phagocytes and natural killer cells, which will lead to lysis of the infected cell (Harper, 1994). Interestingly, not all FIPV- and FECV-infected monocytes/macrophages showed surface expression. Absence of viral proteins on the cell surface has been described for other viruses, such as human cytomegalovirus and equine herpesvirus 1 as a strategy to avoid recognition by the antibody-dependent immune responses (Fish et al, 1996; van der Meulen et al, 2003). Why only half of the infected cells showed surface expression and whether the cells without surface expression are indeed less susceptible towards antibody-dependent complement mediated lysis, remains to be elucidated.

In FIP research, the CrFK cell line is often used to perform in vitro experiments. The results of this study reveal that the CrFK cell line is not the best suitable in vitro model for the study of FIPV and FECV replication at a cellular level. Firstly, the course of infection of FIPV and FECV is similar in CrFK cells, whereas in monocytes there is a clear difference (as there is in vivo). Secondly, a high percentage of infected cells can be reached in CrFK cells (up to 90% of the
inoculated cells) with each cell producing and releasing a relatively small amount of infectious virus (< 10 viruses/cell). In monocytes on the other hand, less than 1% of the cells can be infected, but a single FIPV-infected monocyte releases up to 200 new infectious viruses. Thirdly, CrFK cells showed surface expression in almost all infected cells, in contrast to monocytes, which showed surface expression in only half of the infected cells.

In conclusion, it can be stated that FCoV infection kinetics in vitro are strongly dependent on cellular factors. Monocytes from some cats cannot be infected. If monocytes are susceptible to FCoV infection, then both FIPV and FECV can infect them. However, FECV infections are never sustained and production of viral antigens and progeny virus ceases at 24 hpi. Sustainability of a FIPV infection depends on the origin of the host cells. FIPV production in susceptible monocytes was always 10 to 100 times higher than FECV production. What this variation in susceptibility and sustainability implicates for the development and pathogenesis of FIP and/or FECV in vivo, remains to be elucidated.
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Replication of FCoV in monocytes

References


Nauwynck H (1993) Effect of aging, activation by phorbol myristate acetate and treatment with interferon-γ on the susceptibility of blood monocytes to Aujeszky’s disease virus. PhD thesis, Faculty of Veterinary Medicine, Ghent University


Absence of surface expression of feline infectious peritonitis virus (FIPV) antigens on infected cells isolated from cats with FIP

Summary

Feline infectious peritonitis virus (FIPV) positive cells are present in granulomas and exudates from cats with FIP. These cells belong mainly to the monocyte/macrophage lineage. How these cells survive in infected cats is not known. In this study, FIPV positive cells were isolated from granulomas and exudates of twelve naturally FIPV infected cats and the presence of two immunologic targets, viral antigens and MHC-I molecules, on their surface was determined. The majority of the infected cells (95 ± 5 %) were confirmed to be cells from the monocyte/macrophage lineage. No surface expression of viral antigens was detected on FIPV positive cells. After cultivation of the isolated infected cells, 52 ± 10 % of the infected cells were capable to re-express viral antigens on the plasma membrane. MHC-I molecules were present on all the FIPV positive cells.

In conclusion, it can be stated that in FIP cats, FIPV replicates in cells of the monocyte/macrophage lineage that do not carry viral antigens in their plasma membrane. This indicates that, in vivo, immune-evasion processes that inhibit surface expression of viral proteins occurs. This could allow the infected cells to escape from antibody-dependent cell lysis.
**Introduction**

Feline infectious peritonitis (FIP) is a fatal chronic disease in cats caused by a coronavirus, feline infectious peritonitis virus (FIPV), and characterized by granulomatous lesions formed at the serosas of different organs. Two forms can be distinguished. Cats suffering from the wet or effusive form have exudates in their body cavities. Exudate is absent in the second form, hence the name dry or non-effusive form. FIPV-infected cells are detected in the granulomas and, based on morphology and the granulocyte/monocyte/macrophage marker calprotectin, defined as macrophages (Weiss & Scott, 1981; Kipar et al, 1998). Infected mononuclear cells were also isolated from exudates (Cammarata Parodi et al, 1993; Paltrinieri et al, 1999). By an unknown mechanism, these infected cells succeed in staying alive and transmitting virus to new susceptible cells in the presence of a high concentration of antibodies. Infected cells are normally eliminated by the adaptive immune system through antibody-mediated lysis or cell-mediated lysis. For the antibody-mediated lysis, the presence of antigens on the surface of infected cells is important for the recognition of these cells by antibodies and the subsequent destruction by the immune system (Sissons & Oldstone, 1980). For pseudorabies virus (PRV) and equine herpesvirus type 1 (EHV 1), it has been described that absence of antigens on the surface membrane of infected monocytes, due to antibody-induced internalization or lacking of expression respectively, protects the infected cells from efficient complement-mediated lysis (van der Meulen et al, 2003; Van de Walle et al, 2003; van der Meulen et al, 2006).

In *in vitro* studies with FIPV 79-1146 infected feline monocytes, it was shown that viral antigens are expressed in the plasma membrane in 50% of the infected cells. In these cells, the surface-expressed viral antigens are internalized after addition of antibodies, leaving the plasma membrane of the cell cleared from all visually detectable viral antigens (Dewerchin & Cornelissen et al, 2005; Dewerchin et al, 2006).

Besides antibody-mediated lysis, the adaptive immune system can eliminate virus-infected cells through cell-mediated immunity. A part of the newly synthesized viral proteins in infected cells are disintegrated by proteasomes, the peptides are coupled
to major histocompatibility complex I (MHC-I) molecules and transported to the plasma membrane of the infected cell. This complex is recognized by cytotoxic T-lymphocytes which kill the infected cell. Viruses have developed various ingenious ways to block the MHC-I antigen presentation pathway (Hewitt, 2003). For pseudorabies virus (PRV), it has been described that during antibody-induced internalization of viral glycoproteins in infected blood monocytes, the MHC-I molecules are co-internalized (Favoreel et al, 1999). Absence of MHC-I molecules allows PRV-infected cells to hide from the cell-mediated immunity (Favoreel, 1999). Up till now, it is not known if FIPV affects the MHC-I expression on the surface of FIPV-infected cells in FIP cats.

In the present study, infected monocytes/macrophages of FIP cats were isolated from naturally FIPV infected cats and it was examined if viral antigens are expressed on their plasma membrane and if the expression of MHC-I molecules was inhibited.
Materials and Methods

Cats with naturally occurring FIP. Twelve cats strongly suspected of FIP by clinicians (based on cat profile, clinical signs and blood and/or exudate examination) were used in this study. The sex, age, breed, FCoV antibody titer and type of FIP (effusive or non-effusive) are listed in Table 4.1.

Table 4.1. Breed, age, sex, FCoV antibody titer and pathological form of FIP from the cats enclosed in this study

<table>
<thead>
<tr>
<th>Cat no.</th>
<th>Breed</th>
<th>Age a</th>
<th>Sex b</th>
<th>FCoV antibody titer c</th>
<th>Pathological form</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>British shorthair</td>
<td>10</td>
<td>M</td>
<td>&gt;12800</td>
<td>Non-effusive</td>
</tr>
<tr>
<td>2</td>
<td>Bengal</td>
<td>9</td>
<td>M</td>
<td>1280</td>
<td>Effusive</td>
</tr>
<tr>
<td>3</td>
<td>Persian</td>
<td>9</td>
<td>F</td>
<td>1280</td>
<td>Effusive</td>
</tr>
<tr>
<td>4</td>
<td>Persian</td>
<td>4</td>
<td>M</td>
<td>1600</td>
<td>Effusive</td>
</tr>
<tr>
<td>5</td>
<td>Persian</td>
<td>36</td>
<td>M</td>
<td>6400</td>
<td>Effusive</td>
</tr>
<tr>
<td>6</td>
<td>Exotic shorthair</td>
<td>5</td>
<td>F</td>
<td>640</td>
<td>Effusive</td>
</tr>
<tr>
<td>7</td>
<td>British shorthair</td>
<td>9</td>
<td>F</td>
<td>6400</td>
<td>Effusive</td>
</tr>
<tr>
<td>8</td>
<td>Sphynx</td>
<td>7</td>
<td>M</td>
<td>&gt;12800</td>
<td>Effusive</td>
</tr>
<tr>
<td>9</td>
<td>Sphynx</td>
<td>4</td>
<td>M</td>
<td>1280</td>
<td>Non-effusive</td>
</tr>
<tr>
<td>10</td>
<td>Sphynx</td>
<td>2</td>
<td>F</td>
<td>&gt;12800</td>
<td>Effusive</td>
</tr>
<tr>
<td>11</td>
<td>Persian</td>
<td>42</td>
<td>F</td>
<td>&gt;12800</td>
<td>Effusive</td>
</tr>
<tr>
<td>12</td>
<td>Sphynx</td>
<td>6</td>
<td>F</td>
<td>6400</td>
<td>Non-effusive</td>
</tr>
</tbody>
</table>

a months
b M: male, F: female
c IPMA antibody titer
/ exudate not present (non-effusive form of FIP)

Antibodies. Monospecific, polyclonal antibodies originating from cats infected with serotype II FIPV 79-1146 were kindly provided by Dr. Rottier and Dr. Egberink (Utrecht University, the Netherlands). Polyclonal antibodies against FIPV serotype I were isolated from a cat infected with a serotype I strain. Immunoblotting showed strong reaction with the spike protein of the serotype I strain Black. Both polyclonal antibodies against serotype I and II were purified and biotinylated according to manufacturer’s instructions (Amersham Bioscience, Buckinghamshire, UK). A mixture of both biotinylated antibodies was used in the immunofluorescent stainings (biotinylated anti-FIPV Ab). It was confirmed that this mixture of biotinylated
antibodies was able to stain surface expression of both serotype I as serotype II viruses. Feline polyclonal fluorescein-conjugated antibodies detecting both serotype I and II (anti-FIPV-FITC Ab), a major histocompatibility complex I (MHC-I) marker (CF298A) and a monocyte-macrophage-granulocyte marker (DH59B) were purchased from Veterinary Medical Research and Development (VMRD) (Pullman, Washington, USA).

**Isolation of FIPV positive cells.** Cats were euthanized using 1 ml/1.5 kg Na-pentobarbital (Kela, Hoogstraten, Belgium) and exudates were collected and diluted 1:1 with phosphate-buffered saline (PBS) containing 15 U/ml heparin (Leo, Zaventem, Belgium). Cells present in the exudate were collected by centrifugation at 400×g for 10 minutes at 4°C. Afterwards, tissues with granulomas were collected. Small blocks containing almost just the granulomas were immediately placed in RPMI-1640 at 37°C (Gibco BRL, Merelbeke, Belgium). For isolation of individual cells the small blocks were mechanically separated using 2 needles. The cell suspension was then centrifuged at 400×g for 10 minutes at 4°C.

**Characterization of FIPV positive cells.** The obtained cell suspensions from the exudates and the tissues with granulomas from cat 1 to 9 were each divided in three parts on which different stainings in suspension were performed. The cells from the granulomas and the exudate of cat 2 were stained together. The first staining was performed to determine the viability and the monocyte/macrophage nature of the FIPV positive cells. Since the marker DH59B also detects granulocytes, besides macrophages and monocytes, the morphology of the nucleus was taken into account to determine whether the cells belonged to the monocyte/macrophage lineage. The second staining was performed to detect if viral antigens were present on the surface of FIPV positive cells. The third staining was performed to determine the presence of MHC-I molecules on the surface of FIPV positive cells and the effect on the viability of the cells. The latter staining was only performed for cats 6, 7, 8 and 9. The different staining steps and used antibodies and conjugates are given in Table 4.2.
After staining, cells were mounted on microscope slides using glycerin-PBS solution (0.9/0.1, vol/vol) with 2.5% 1,4-diazabicyclo(2,2,2)octane (Janssen Chimica, Beerse, Belgium) and analyzed using a Leica DM RBE fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**Cultivation of FIPV positive cells.** The isolated cells from cats 10, 11 and 12 were cultured in a 24-well plate on a glass coverslip for 0, 2, 4 and 6 hours. At each time point, the immunofluorescence staining for detection of surface-expressed viral antigens was performed (staining 2). After staining, the glass coverslips were mounted on microscope slides and analyzed by fluorescence microscopy.

**Control: possible steric hindrance of antibodies already bound to antigens.** In FIPV-infected cats high levels of anti-FCoV antibodies are found. If viral antigens are present on the surface of infected cells, they will be bound by these antibodies. This control was performed to determine whether the antibodies used in staining 2 (biotinylated anti-FIPV Ab) undergo steric hindrance of antibodies already bound to antigens. Therefore, CrFK cells were infected with FIPV 79-1146 (type II) and the surface-expressed viral antigens were blocked by strain specific polyclonal antibodies. Then, the biotinylated anti-FIPV Ab were added and visualized by streptavidin-Texas Red to determine if they were still able to detect the surface-expressed viral antigens. This was also determined on fcwf cells infected with FIPV Black (type I) with blocked surface-expressed viral antigens by type I polyclonal antibodies.
Statistical analysis. Results were analyzed with the Wilcoxon signed ranks test. Statistical analyses were performed with SPSS 11.0 (SPSS Inc. Chicago, Illinois, USA).

Table 4.2: Antibodies and conjugates used in the different staining steps for the identification of macrophage/monocytic cells and viability (staining 1), detection of surface expressed viral antigens (staining 2) and presence of MHC I (staining 3) on isolated cells.

<table>
<thead>
<tr>
<th>Different staining steps (1-6)</th>
<th>Staining 1</th>
<th>Staining 2</th>
<th>Staining 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Viability staining</td>
<td>EMA&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>/</td>
<td>EMA&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>2. Fixation</td>
<td>Formaldehyde 1%</td>
<td>Formaldehyde 1%</td>
<td>Formaldehyde 1%</td>
</tr>
<tr>
<td>3. Cell surface staining</td>
<td>Monocyte marker (DH59B) +</td>
<td>Biotinylated anti-FIPV Ab +</td>
<td>MHC I marker (CF298A) +</td>
</tr>
<tr>
<td></td>
<td>Goat anti-mouse-FITC&lt;sup&gt;b&lt;/sup&gt; (green)</td>
<td>Streptavidin-Texas Red&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Goat anti-mouse-FITC&lt;sup&gt;b&lt;/sup&gt; (green)</td>
</tr>
<tr>
<td>4. Permeabilization</td>
<td>Triton X-100&lt;sup&gt;c&lt;/sup&gt; 0.1%</td>
<td>Triton X-100&lt;sup&gt;c&lt;/sup&gt; 0.1%</td>
<td>Triton X-100&lt;sup&gt;c&lt;/sup&gt; 0.1%</td>
</tr>
<tr>
<td>5. Cytoplasmic staining</td>
<td>Biotinylated anti-FIPV Ab +</td>
<td>Anti-FIPV-FITC Ab (green)</td>
<td>Biotinylated anti-FIPV Ab +</td>
</tr>
<tr>
<td></td>
<td>Streptavidin-Alexa fluor&lt;sup&gt;a&lt;/sup&gt; 350&lt;sup&gt;b&lt;/sup&gt; (blue)</td>
<td>Streptavidin-Alexa fluor&lt;sup&gt;a&lt;/sup&gt; 350&lt;sup&gt;b&lt;/sup&gt; (blue)</td>
<td></td>
</tr>
<tr>
<td>6. DNA staining</td>
<td>/</td>
<td>Hoechst 33342&lt;sup&gt;b&lt;/sup&gt;</td>
<td>/</td>
</tr>
</tbody>
</table>

<sup>a</sup> Ethidium mono-azide bromide which specifically stains the nuclei of dead cells (red)

<sup>b</sup> Molecular Probes (Eugene, Oregon, USA)

<sup>c</sup> Sigma-Aldrich GmbH (Steinheim, Germany)
Results

Isolation and characterization of FIPV positive cells. FIPV positive cells were found in cell suspensions from exudates and granulomas in all cats. The percentage of FIPV positive cells varied from <1 to 10% (Table 4.3). The majority of the FIPV positive cells (95 ± 5 %) belonged to the monocyte/macrophage lineage (mononuclear and DH59B positive) (Figure 4.1, lane A). Less than 1% of the FIPV positive cells showed a polymorphonuclear nucleus. The viability staining (staining 1) showed that the percentage of dead FIPV positive monocytes in granulomas was significantly higher than the control cells (FIPV negative, DH95B positive cells) (p<0.1), whereas no overall difference was observed between FIPV positive monocytes and the control cells in exudates (Table 4.3).

Table 4.3: Quantification, identification and determination of viability of FIPV positive (+) cells isolated from granulomas and exudates of 9 FIP cats

<table>
<thead>
<tr>
<th>Cat n°</th>
<th>Origin of the cells</th>
<th>FIPV + cells (%) of total cells</th>
<th>Monocytes/macrophages (%) of FIPV + cells</th>
<th>Dead cells (%)</th>
<th>FIPV + monocytes</th>
<th>FIPV - monocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Granulomas</td>
<td>5</td>
<td>98</td>
<td>51</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Granulomas + exudate</td>
<td>10</td>
<td>96</td>
<td>18</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Granulomas</td>
<td>1</td>
<td>92</td>
<td>2</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exudate</td>
<td>1</td>
<td>90</td>
<td>10</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Granulomas</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exudate</td>
<td>&lt;1 b</td>
<td>86</td>
<td>0</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Granulomas</td>
<td>1</td>
<td>100</td>
<td>38</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exudate</td>
<td>1</td>
<td>100</td>
<td>8</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Granulomas</td>
<td>1</td>
<td>98</td>
<td>21</td>
<td>15</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exudate</td>
<td>&lt;1 b</td>
<td>98</td>
<td>12</td>
<td>21</td>
<td></td>
</tr>
<tr>
<td>7</td>
<td>Granulomas</td>
<td>1</td>
<td>86</td>
<td>64</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exudate</td>
<td>1</td>
<td>90</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>Granulomas</td>
<td>&lt;1 b</td>
<td>100</td>
<td>0</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Exudate</td>
<td>1</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>Granulomas</td>
<td>&lt;1 b</td>
<td>100</td>
<td>7</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

a mononuclear and DH59B positive

b <1: antigen positive cells present but only a few per slide

ND not detected: no antigen positive cells on the slide
Staining 2 (detection of surface-expressed viral antigens) revealed that no infected cells showed expression of viral antigens on their surface (Figure 4.1, lane B). The results of the MHC-I staining (staining 3) in isolated cells of cats 6, 7, 8 and 9 showed that MHC-I expression was present on $98 \pm 3\%$ of the FIPV positive cells (Figure 4.1, lane C). No difference in amount of MHC-I expression was observed between live and dead cells.

Figure 4.1: Surface expression of the monocyte marker (A); absence of surface expression of viral proteins (B); surface expression of MHC I (C) on cytoplasmic FIPV positive cells.
**Cultivation of FIPV positive cells.** After cultivation of the FIPV positive cells, viral antigens were re-expressed on the plasma membrane as soon as 2 hours post-seeding. Re-expression only occurred in 52 ± 10 % of infected cells (Figure 4.2).

![FIPV proteins expression](image)

**Figure 4.2:** Re-expression of viral proteins on the plasma membrane after cultivation of FIPV positive cells isolated from three cats with naturally occurring FIP: immunofluorescence pictures (A); kinetics (B).

**Control: possible steric hindrance of antibodies already bound to antigens.** Although surface-expressed viral antigens on CrFK cells and fcwf cells were bound by antibodies, the biotinylated anti-FIPV antibodies were still able to detect the viral antigens.
Discussion

In this study, FIPV positive monocytes were isolated from naturally FIPV infected cats and the presence of two immunologic targets was examined. It was shown that these cells do express MHC-I molecules on their surface, but no surface-expressed viral antigens were detected. Approximately half of these cells were capable of re-expressing viral antigens on their surface during cultivation.

The presence of viral antigens on the cell surface of infected cells is important for the recognition and elimination of infected cells by the immune system. Binding of virus-specific antibodies to viral proteins present on the surface, makes infected cells recognizable for the classical complement pathway, phagocytes and natural killer cells, which will lead to lysis of infected cells (Harper, 1994). In this study, viral antigens were not detected on the surface of FIPV positive cells isolated from 9 cats with FIP. This could imply that the FIPV positive cells may remain “invisible” for the humoral immune system and continue the production of progeny virus without being eliminated. It is not known which mechanism lies behind the absence of surface-expressed viral antigens. The results of the cultivation experiment demonstrated that about half of the infected cells do not express viral antigens on their surface. This indicates that the viral proteins are retained inside the cell. This observation is consistent with the in vitro findings of Dewerchin & Cornelissen et al (2005). They showed that 50% of FIPV 79-1146 infected monocytes do not express viral antigens on the plasma membrane.

The absence of viral antigens on the surface of isolated FIPV positive cells that are capable of expressing viral antigens can be due to the fact that virus specific antibodies bind to the antigens and as a consequence the viral antigens are internalized. Another possibility is antibody-induced capping of viral antigens and extrusion from the cell. Since the antibody induced internalization has been described in in vitro infected monocytes it most likely occurs in vivo as well (Dewerchin et al, 2006). The fact that no surface-expressed viral antigens could be demonstrated with the used staining is not due to steric hindrance of antibodies already bound to these antigens in the cat.
Next to antibody-mediated elimination of virus-infected cells, cytotoxic T-lymphocytes (CTLs) are also capable of killing infected cells. During an infection, viral peptides are loaded on MHC-I molecules and transported to the plasma membrane. This complex may be recognized by CTLs which leads to killing of the infected cell. In this study, the presence of MHC-I on FIPV positive cells was analyzed. On all FIPV positive cells MHC-I was present, showing that no internalization or retention of the MHC-I molecules occurs. However, with the used techniques, it was not possible to quantify the number of MHC-I molecules and to determine whether the MHC-I molecules were loaded with FIPV peptides.

Since no viral antigens are present on the plasma membrane, it can be stated that antibody-mediated lysis is inhibited. The expression of MHC-I molecules is not inhibited in infected cells, indicating that the cellular immunity may still be able to lyse the infected cell, if viral peptides are presented. It is generally accepted that a strong cellular immunity enables the cat to overcome infection (Pedersen & Black, 1983; Hayashi et al, 1983; Weiss & Cox, 1989). However, it has also been postulated that, during a chronic FIPV infection, the cell-mediated lysis is inhibited due to apoptosis and T-cell depletion caused by soluble mediators released during infection (Haagmans et al, 1996; de Groot-Mijnes, 2005). Taking into account all these observations, it becomes clear that the outcome of a FIPV infection is a complicated interaction of the immune system and the virus. One thing is sure, the humoral immune response is not able to protect the cat against progression of viral replication and consequently of disease. The precise role of the cellular immune response in protection needs to be further investigated.

In conclusion, it can be stated that cytoplasmic FIPV-infected cells do not show surface-expressed viral antigens \textit{in vivo} which may make them invisible for the humoral immune response. In contrast, MHC-I molecules are abundantly present on their surface.
Acknowledgments

We are grateful to Dr. Rottier and Dr. Egberink for supplying antibodies. We thank doctors of veterinary medicine Burrick, Reybroeck, van de Werf and Criel and the “Department of Medicine and clinical biology of small animals” of the Faculty of Veterinary Science for their co-operation. E. Cornelissen and H.L. Dewerchin were supported by the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT-Vlaanderen). E. Van Hamme was supported by a doctoral grant from the special research fund of Ghent University.
References


Absence of antibody-dependent, complement-mediated lysis of feline infectious peritonitis virus infected cells

Adapted from:
Summary

Cats infected with virulent feline coronavirus which causes feline infectious peritonitis (FIP) usually succumb to disease despite high antibody concentrations. One of the mechanisms that can help resolving infection is antibody-dependent, complement-mediated lysis (ADCML) of infected cells. ADCML consists of virus-specific antibodies that bind to cell surface expressed viral proteins which results in complement activation and cell lysis. The objective of this study was to determine the sensitivity of FIP-virus (FIPV) infected cells towards ADCML and to examine the role of the accessory proteins 3abc and 7ab in this process. ADCML assays, using FIPV strain 79-1146 and its deletion mutant strain Δ3abc/Δ7ab, were performed on (i) CrFK cells that show surface-expressed viral antigens (ii) monocytes without surface-expressed viral proteins due to retention and (iii) monocytes with surface-expressed viral proteins since the antibody-mediated internalization of these proteins was blocked. As expected, no ADCML was detected of the monocytes without surface-expressed viral antigens. Surprisingly, no lysis was observed in the CrFK cells and the monocytes that do show surface-expressed viral proteins. Controls showed that the ADCML assay was functional though. These experiments prove that FIPV can employ another immune evasion strategy against ADCML (besides preventing surface expression): the inhibition of complement mediated lysis. This new evasion strategy is not attributed to the group specific proteins since lysis of cells infected with FIPV Δ3abc/Δ7ab was not detected.
Absence of ADCML of FIPV-infected cells

Introduction

Feline infectious peritonitis (FIP) is a fatal disease, characterized by fibrinous-granulomatous serositis often with protein-rich effusions in body cavities, granulomatous-necrotising phlebitis and periphlebitis and granulomatous inflammatory lesions in several organs (Weiss and Scott, 1981a, 1981b; Kipar et al, 1998, 2005). The causative agent is a virulent form of the feline coronaviruses (FCoVs) belonging to the family Coronaviridae, order Nidovirales. In vivo, monocytes and tissue macrophages are the target cells and play a central role in the development of the lesions (Kipar et al, 2005). These infected cells should be excellent targets for the immune system to fight the infection. However, in most FIP cases the immune response is not protective and the cat succumbs to the infection. The cell-mediated immunity is thought to be important in control and clearance of the FIP virus (FIPV) infection if there is an efficient first response to the infection. The humoral immune response is thought to be not protective (Pedersen, 1987). High concentrations of neutralizing antibodies are present in cats with end-stage FIP and no difference is seen in the antibody concentration and fluctuations between survivors and non-survivors after a FIPV infection (de Groot-Mijnes et al, 2005).

In general, virus-specific antibodies can help to resolve infection by antibody-mediated lysis of infected cells via cytolytic immune cells with Fc receptor (like NK cells, macrophages or neutrophils) or via complement (Sissons and Oldstone, 1980). The complement system is an immunological defense system and plays a role in both the innate and the adaptive immune response against invading pathogens. Complement consists of serum and membrane-bound proteins which, once activated, can trigger a biochemical cascade of reactions contributing to the eradication of pathogens (Blue et al, 2004). Important complement effector functions are opsonization of pathogens, cytolysis and promoting host inflammatory responses (anaphylatoxin and chemotaxin production) (Janeway et al, 2005). In viral infections, the complement system can be activated by free virus particles and virus-infected cells. Complement can inactivate free virus in the presence or absence of antibodies. Opsonization of the virus with complement proteins can promote phagocytosis, virolysis and interference with attachment, internalization or uncoating of the virions (Hirsch, 1982). Cells infected with enveloped viruses can be
lysed by complement in the presence of antibodies if newly synthesized viral glycoproteins are expressed at the plasma membrane of the infected cell. Virus-specific antibodies can then bind to these surface expressed proteins and thereby activate the complement system. Eventually, this results in cell death (Sissons and Oldstone, 1980). This process is called antibody-dependent, complement-mediated lysis (ADCML).

Recently, we described for FIPV two processes that inhibit the expression of viral proteins at the plasma membrane of in vitro infected monocytes. Namely, the retention of viral proteins in infected cells and the antibody-mediated internalization of surface expressed viral proteins. Both processes result in the clearance of all detectable viral antigens from the plasma membrane of infected cells (Dewerchin et al, 2005; Dewerchin et al, 2006). FIPV-infected monocytes/macrophages isolated from naturally infected cats do not express viral proteins at their plasma membrane either (Cornelissen et al, 2007). Absence of viral proteins in the plasma membrane of infected monocytes can protect the infected cells from efficient ADCML. This has been described for pseudorabies virus (PRV), equine herpesvirus type 1 (EHV-1) and porcine reproductive and respiratory syndrome virus (PRRSV) (van der Meulen et al, 2003; Van de Walle et al, 2003; Costers et al, 2006).

The objective of this study was to determine if (i) absence of surface-expressed viral antigens can protect the infected cell against ADCML and (ii) if there is efficient ADCML of FIPV-infected cells that do show surface-expressed viral antigens. If so, this would open treatment possibilities based on inhibiting antibody-mediated internalization of surface-expressed viral antigens. Furthermore, the role of the accessory proteins 3abc and 7ab was assessed in this context. The role of these accessory proteins in the FIPV pathogenesis is not yet fully understood, but deletion of these strains gives an attenuating effect in vivo (Haijema et al, 2004). This may indicate that an immune evasion process is lost due to this deletion.
Materials and Methods

Viruses. FIPV serotype II strain 79-1146 and its attenuated deletion mutant virus strain FIPV \( \Delta 3abc/\Delta 7ab \) were used. The deletion mutant strain is the FIPV strain 79-1146 from which the open reading frames 3abc and 7ab were deleted, using reverse genetics (Haijema et al, 2004). Both viruses were kindly provided by Dr Rottier (Faculty of Veterinary Medicine, Utrecht University, The Netherlands).

Cells. CrFK cells were seeded in 6-well plates (Nunc A/S, Roskilde, Denmark) and cultivated in MEM-medium containing 5% fetal bovine serum, 2% lactalbumine, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin and 0.1 mg/ml kanamycin. Peripheral blood monocytes were isolated from feline coronavirus, feline leukemia virus and feline immunodeficiency virus negative cats as described previously (Dewerchin et al, 2005). They were cultivated in 6-well plates in RPMI 1640-medium containing 10% fetal bovine serum, 0.3 mg/ml glutamine, 100 U/ml penicillin, 0.1 mg/ml streptomycin, 0.1 mg/ml kanamycin, 10 U/ml heparin, 1mM sodium pyruvate and 1% non-essential amino-acids 100x (Gibco BRL, Merelbeke, Belgium).

Antibodies and complement. The FIPV-specific polyclonal antibodies (pAbs) originated from cats infected with FIPV 79-1146 and were provided by Dr Rottier (Utrecht University, the Netherlands). FIPV-negative pAbs were purified from FIPV-negative serum derived from an FCoV negative cat (IPMA antibody titer < 20). Both pAbs had been purified using protein A-Sepharose (Amersham Bioscience, Buckinghamshire, UK). Non-inactivated serum of a FCoV-negative cat was used as a source of complement.

ADCML assays with FIPV. Cells were infected with FIPV serotype II strain 79-1146 or with its attenuated deletion mutant virus strain FIPV \( \Delta 3abc/\Delta 7ab \) at a multiplicity of infection of 1. The CrFK cells and the monocytes were mechanically detached (by gently pipetting up and down) from the wells at 18 hours post inoculation (hpi) and 12 hpi, respectively, to perform the assays in suspension. The cells were incubated for 1 hour with FIPV-specific pAbs (0, 0.9 and 1.8 mg/ml) or
FIPV-negative antibodies (1.8 mg/ml). The cells were washed and incubated with 5% complement. Then, the cells were stained with ethidium monoazide bromide (EMA) (Molecular Probes, Eugene, Oregon, USA) to label dead cells, fixed with 3% paraformaldehyde (Sigma-Aldrich GmbH, Steinheim, Germany), permeabilized with 0.1% saponin (Sigma-Aldrich GmbH) and stained with specific monoclonal antibodies (mAbs) against FIPV nucleocapsid (N) protein and membrane (M) protein, followed by FITC-labeled goat anti-mouse IgG (Molecular Probes) to identify FIPV-infected cells. The mAbs recognizing the M and N protein were produced and characterized in our laboratory. Nuclei were stained with Hoechst 33342 (Molecular Probes). Dead infected cells were counted using fluorescence microscopy. For the monocytes, the antibody-induced internalization was inhibited by pre-treatment for 30 minutes with myosin light chain kinase inhibitor (ML7) (Calbiochem, San Diego, California, USA) and inclusion of the inhibitor during antibody and complement incubation (Dewerchin, 2008).

**Detection of membrane bound antibodies.** Since antibodies must be bound to the cell before ADCML can occur, an immunofluorescent staining was performed to determine the presence of the antibodies on the surface of the infected cells. Cells were fixed with 3% paraformaldehyde after incubation with FIPV-specific pAbs. Antibodies were stained with FITC-labeled goat anti-cat IgG (Sigma-Aldrich GmbH). After permeabilization with 0.1% saponin, infected cells were stained with N- and M-specific mAbs and Texas Red-labeled goat anti-mouse IgG (Molecular Probes). Nuclei were stained with Hoechst 33342 (Molecular Probes).

**Determination of the functionality of the ADCML assay.** Two control assays were performed to verify the functionality of the ADCML assay: (i) a FIPV neutralization assay to detect a higher neutralization in the presence of complement in order to confirm the activity of the feline complement in combination with the FIPV-specific pAbs and (ii) an ADCML assay on pseudorabies virus (PRV)-infected feline monocytes and CrFK cells to confirm the activity of the feline complement, the sensitivity of the cells to ADCML and to exclude possible interference of the used media.
For the neutralization assay, 2-fold dilution series of FIPV-specific polyclonal antibodies were incubated for 1 hour with FIPV 79-1146 at a titer of 100, 1000 and 10 000 TCID₅₀. Feline complement (5%) was added for 1 hour. The pAbs-virus mixture, with or without complement, was brought on felis catus whole foetus (fcwf) cells for 1 hour. The neutralization titer was determined on the basis of formation of cytopathic effect.

The ADCML assay on PRV-infected feline monocytes and CrFK cells was performed as described above using PRV strain Kaplan, feline pAbs against PRV (derived from a Geskypur (Merial) vaccinated FCoV-negative cat, according to manufacturer’s instructions), feline complement and FITC-labeled PRV-specific pAbs to identify infected cells. It has been described that PRV-infected porcine monocytes with surface-expressed viral antigens are sensitive to ADCML (Van de Walle et al, 2003).

**Statistical analysis.**

Triplicate ADCML assays were performed and results were compared using the Friedman Test from the SPSS software package (version 12.0; SPSS Inc., Chicago, Illinois, USA).
Results

ADCML assays with FIPV. The results of the ADCML with FIPV 79-1146 and the deletion mutant virus strain FIPV Δ3abc/Δ7ab are given in Figure 5.1. With both viruses and both cell types, the percentages of dead FIPV-infected cells in the ADCML assay with virus-specific antibodies were not significantly different from those in the assay without antibodies (p ≤ 0.05). Also no significant difference was seen with the ADCML assay with FIPV-negative antibodies.

![Antibody concentrations](image)

Figure 5.1: Antibody-dependent, complement-mediated lysis (ADCML)-assay on CrFK cells and monocytes infected with FIPV strain 79-1146, its deletion mutant FIPV Δ3abc/Δ7ab and pseudorabies virus (PRV) strain Kaplan as a control. Different concentrations of virus-specific antibodies were used.

Detection of membrane bound antibodies. The presence of FIPV-specific antibodies on the surface of infected cells was similar for FIPV 79-1146 and FIPV Δ3abc/Δ7ab. Of the infected CrFK cells, 99 ± 0.3% showed surface-bound antibodies. Formation of small aggregates was seen in approximately one-fourth of these cells (Figure 5.2A) while the other cells showed larger aggregates (patches) (Figure 5.2B). Of the infected monocytes, 53 ± 1% showed surface-bound antibodies. Approximately two thirds of these cells showed small antibody
Absence of ADCML of FIPV-infected cells

aggregates (Figure 5.2C) while the remaining cells showed a more homogeneous antibody distribution (Figure 5.2D). This means that cells are protected against ADCML, irrespective of the expression of viral proteins at their surface.

Figure 5.2: Distribution of FIPV-specific antibodies on FIPV-infected cells. CrFKs showed small (A) or larger (B) antibody aggregates. The monocytes showed either small antibody aggregates (C) or more homogenous antibody distribution (D). The pictures show an image of the cell constructed by super-imposing the images obtained at different sections throughout the cell.

Determination of the functionality of the ADCML assay.

The FIPV neutralization assay with and without complement showed that the complement was effective in enhancing the neutralization of FIPV. In the presence of complement the virus neutralization titer was 2 times and 64 times higher for 1000 and 10 000 TCID_{50} of FIPV 79-1146, respectively. No enhancement was seen with 100 TCID_{50} of FIPV 79-1146.

The ADCML assay with PRV showed that the cells were sensitive to ADCML. The ADCML was effective for both CrFK cells and feline monocytes. For both cell types there was a significant rise in the percentage of dead cells with higher antibody concentrations (p ≤ 0.05; Friedman Test) (Figure 5.1).
Discussion

The humoral immune system is activated during a FIPV infection, but the antibodies formed seem to be ineffective in eliminating virus and virus-infected cells. For FIPV-infected cells, antibody-mediated internalization of plasma membrane expressed viral proteins has been described (Dewerchin et al, 2006). This internalization, resulting in the absence of viral proteins in the plasma membrane, can protect the infected cells from efficient ADCML. Inhibiting this internalization process, resulting in infected cells being recognizable by antibodies, could be a part of a treatment protocol for FIP cats. Unfortunately, results of this study show that even if there are viral proteins present on the plasma membrane, no lysis does occur through ADCML. However, if antibodies bind to the surface expressed viral proteins, cell lysis may still occur via other cell lysis mechanisms e.g. via antibody-dependent, cell-mediated cytotoxicity. In this mechanism, lysis is performed by activated natural killer cells, neutrophils, monocytes or macrophages.

Lysis of virus-infected cells by antibodies and complement requires viral proteins that are expressed in a form and configuration recognizable by antibodies that can bind complement (Hirsch, 1982). The FIPV-specific antibodies used are able to bind complement since virus-neutralization was higher in the presence of complement. This higher neutralization can be due to virolysis, agglutination of virus-antibody-complement complexes or coating of the virus with complement components which can interfere with the binding of the virus to target cells or alter the surface charge of the virus (Hirsch, 1982; Lachmann and Davies, 1997). For example, it could be possible that binding of complement to the Fc portion of the antibody inhibits antibody dependent enhancement of infectivity (ADEI), a mechanism which is described in vitro (Hohdatsu et al, 1991). The fact that neutralizing antibodies are present in a cat with FIP, together with the observed enhancement of neutralization by complement in this study, indicates that cell free virus does not play an important role in the pathogenesis of FIP (de Groot-Mijnes, 2005).

For the effectiveness of the ADCML, the amount of bound antibodies is also important. The more antibodies that are bound on the surface of infected cells, the higher the percentage of lysed cells is (Joseph et al, 1976). All FIPV-infected CrFKs showed bound antibodies on their surface. The formation of patches is not likely to
have an influence on the ADCML assay. It has been described for measles virus infected cells that redistribution of viral antigens has no influence on the effectiveness of the ADCML (Perrin et al, 1976). Half of the infected monocytes showed bound antibodies on their surface, which is consistent with previously published results (Dewerchin et al, 2005). As expected, the monocytes without surface expressed viral proteins were protected against ADCML, but also no ADCML was seen of the monocytes with surface expressed viral proteins. The possibility that insufficient amounts of antibodies were bound on infected CrFKs and monocytes to have a detectable effect of ADCML is unlikely, but cannot be completely excluded.

It seems that FIPV can employ another virus complement evasion strategy in addition to the already described retention of viral proteins in the cytoplasm of infected cells and the antibody-mediated internalization of plasma membrane expressed viral proteins. This additional evasion strategy cannot be attributed to the accessory or group specific proteins 3abc and 7ab since lysis of cells infected with the double mutant strain FIPV Δ3abc/Δ7ab was not detected. These genes encode for proteins that are not necessary for virus growth and infection. Their deletion has an attenuating effect on the virulence of the virus in cats (Haijema et al, 2004). The basis of the attenuation is not known, but there is no correlation with the efficacy of the ADCML, as shown in the present study. This new immune evasion strategy has to be attributed to the structural proteins or the non-structural proteins of ORF 1ab and can be direct via viral proteins or indirect by means of cellular proteins that regulate the complement cascade. Various virus complement evasion strategies have been described for other viruses. Viruses can express proteins with Fc receptor activity that can inhibit the binding of complement to the antibody-antigen complex. The glycoprotein gE expressed by herpes simplex virus (HSV) has an Fc receptor activity and protects against antibody and complement-mediated lysis, both in vitro and in vivo (Adler et al, 1978; Lubinski et al, 2002). The glycoprotein complex gE-gI expressed by PRV also possesses Fc receptor activity and aids in avoiding efficient ADCML of PRV-infected monocytes in vitro (Van de Walle et al, 2003). For FIPV, the presence of an Fc receptor activity has never been studied but the spike proteins of other coronaviruses, namely mouse hepatitis virus (MHV), bovine coronavirus (BCV) and transmissible gastroenteritis virus (TGEV) display Fc
receptor activity (Oleszak et al, 1995). Another possibility is that the virus encodes proteins with functional similarities to complement control proteins, inhibitors of the complement cascade. No viral complement control proteins have been described for coronaviruses but several have been described for poxviruses and herpesviruses (Favoreel et al, 2003; Bernet et al, 2003). For FIPV, this would imply that the spike, membrane and envelope protein or the non-structural proteins encoded by ORF 1 should exhibit complement control protein activity, like binding to or accelerating the decay of certain complement factors. Finally, it is possible that the virus induces an upregulation of host complement control factors in the infected cell. This has been described for human cytomegalovirus (HCMV) where upregulation of complement regulator CD55 protected infected cells from complement-mediated lysis (Spiller et al, 1996).

FIPV occurs in two types (type I and II) with type I prevailing in the field (Pedersen, 2009). Both types can cause clinical FIP and evade the immune system of the cat. The results in this study were obtained with a type II strain. Whether cells infected with a type I strain also show inhibition of complement mediated lysis has to be determined. A different outcome is possible since this evasion strategy could be attributed to the S protein and the main differences between type I and II are found in this protein (Herrewegh et al, 1998).

In conclusion, it can be stated that FIPV-infected cells are protected against ADCML, both the cells with and without surface expressed viral proteins. It appears that during evolution, FIPV has become a master in disguise, exhibiting several immune evasion mechanisms to avoid clearance of infected cells by the humoral immune response.
Absence of ADCML of FIPV-infected cells

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References


Costers S, Delputte P, Nauwynck H (2006) Porcine reproductive and respiratory syndrome virus-infected alveolar macrophages contain no detectable levels of viral proteins in their plasma membrane and are protected against antibody-dependent, complement-mediated cell lysis. J Gen Virol 87(8), 2341-2351


Chapter 6

General discussion
Feline enteric coronavirus (FECV) is an avirulent variant of feline coronaviruses and is endemic in the cat population. Occasionally, it can mutate to feline infectious peritonitis virus (FIPV), the virulent variant which causes the disease feline infectious peritonitis (FIP). FIP is for veterinarians and cat owners a difficult and in most cases a hopeless situation since no effective treatment and prevention is available.

Although FECV and FIPV act pathogenically very differently, they are genetically rather similar; more than 98% of the genome is identical in isolates from the same environment (Vennema et al, 1995). It is currently not fully clarified why these virus variants manifest so differently \textit{in vivo}. FECV replicates mainly in the enterocytes and can occasionally cause a mild diarrhea and vomiting. The infection is rarely fatal (Pedersen et al, 1981; Kipar et al, 1998). FECV is also capable of spreading throughout the body in blood monocytes and reaching different organs, without causing any systemic clinical signs (Gunn-Moore et al, 1998; Meli et al, 2004; Simons et al, 2005). In contrast, FIPV can spread throughout the body through the blood monocyte, its main target cell, and this results in a highly fatal fibrinous-granulomatous serositis (Kipar et al, 2005).

The virus-infected cell, more specifically the virus-infected monocyte, is important in the pathogenesis of the feline coronaviruses. This infected blood monocyte should be an ideal target for the immune system to stop or inhibit the virus infection. The immune response is activated during FIPV infection, but the infected monocyte is still capable of staying alive and transmitting virus to new susceptible cells. This indicates that mechanisms inhibiting the actions of the immune system are present.

In this thesis, the interaction of the feline coronaviruses with the blood monocyte was studied to determine if there was a difference between FIPV and FECV replication \textit{in vitro} that could explain the differences \textit{in vivo}. Also, the presence of immunological targets on the infected cell were studied both \textit{in vitro} as \textit{ex vivo} to determine whether immune evasion processes on the level of the infected cell are present.

**Feline coronavirus replication**

The infection kinetics in peripheral blood monocytes did not only show a difference between FIPV and FECV but, to our surprise, it also showed a difference between
cats: 3 different infection patterns were detected. In the first two patterns both FECV and FIPV could initially infect the monocytes, but in the first pattern only the FIPV infection was progressive while the FECV infection was not sustained. In the second pattern both FIPV and FECV were not sustained. Overall, it was seen that the total virus production (viral load) was up to 100 times higher in FIPV-infected monocytes compared to FECV-infected monocytes. In the third pattern the monocytes were unsusceptible for both FECV or FIPV infection.

The observed difference in lack of sustainability and long-term production between the viruses could be correlated with the different pathogenesis of these viruses in vivo. Both FIPV and FECV can infect monocytes, indicating that both viruses can reach the blood stream. Only with FIPV, systemic effects and clinical signs are seen because of the higher viral load and progressive infection. Similar replication characteristics have also been detected in other in vitro experiments using feline peritoneal macrophages and feline bone-marrow derived macrophages (Stoddart and Scott, 1989; Rottier et al., 2005). The mutation responsible for this in vitro difference in the replication cycle is probably solely located in the S gene, more specifically in the membrane-proximal domain (involved in cell entry) and not the receptor-binding domain of the spike protein (Rottier et al, 2005). The hypothesis that FCoV virulence is associated with a higher viral load is further supported by in vivo results showing that FCoV replication in blood monocytes mainly occurs in cats with FIP and that higher viral loads are present in haemolymphatic tissues of cats with FIP in comparison to healthy FCoV positive cats (Simons et al, 2005; Kipar et al, 2006). Also other mutations, besides the one in the S protein, have been proposed to be associated with change in FCoV virulence, namely mutations in the group-specific genes 3c, 7a and 7b (Vennema et al, 1998; Kennedy et al, 2001). Viruses lacking these group-specific genes can infect the host without any pathology (Haijema et al, 2004). Overall, these findings suggest that mutation from FECV to FIPV involves multiple mutations, both in the S protein and in the group-specific genes.

Whether there is a correlation between the different patterns seen in vitro and the different clinical outcomes seen after FCoV infection (overcoming the infection, chronic carrier and shedder, FIP development and resistance to infection) is still a question. It is also not known if a cat would always show one specific pattern in vitro in their monocytes or if other patterns could be presented after some time. The
first situation would indicate that host genetic factors are important, while the second would indicate that the differentiation/activation status of the monocytes and the cat’s immune response play a role. Host genetic factors have been suggested to play a role in the outcome of FCoV infections and inherited genetic susceptibility has been described for FIP (Foley and Pedersen, 1996; Addie et al, 2003). The most interesting correlation to investigate would be between the in vitro resistance detected in this thesis and the resistance to FCoV seen in vivo. A genetic correlation could open perspectives for screening and selection of cats not susceptible to FIP and the development of increased resistance through selective breeding.

The differentiation/activation status of the cell can also play a role. In this thesis, the FIPV infection rate of feline blood monocytes was lower than 1%. A low infection rate has also been described in peritoneal macrophages, whereas higher rates were seen in bone-marrow derived macrophages (Stoddart and Scott, 1989; Rottier et al, 2005). This indicates that specific cellular properties are necessary for infection. Our FIPV research group demonstrated that virus binding and uptake takes place in almost all monocytes indicating that the mechanism behind the resistance of most monocytes/macrophages must lie in inhibition of genome release and/or translation (Van Hamme et al, 2007). The percentage of infected cells in the blood of cats with FIP is unknown. FCoV mRNA has been detected in blood monocytes and infected monocytes have been demonstrated in situ in vessels in a cat with FIP (Simons et al, 2005; Kipar et al, 2005). During our study, no FIPV positive cells were detected in the isolated peripheral blood monocytes from cats with FIP (author’s unpublished data). This indicates that also in vivo the percentage of infected blood monocytes is low. However, the percentage of infection in isolated peripheral blood monocytes can be an underestimation due to the fact that blood monocytes, once infected, are activated and attach to endothelial cells and leave the blood circulation (Kipar et al, 2005). Infected cells are detected in granulomas and exudates in a cat with FIP. These monocytes/macrophages can either be infected monocytes that left the blood stream or monocytes/macrophages that were attracted to the site of infection and became infected. The latter cells could have a higher infection rate due to their different differentiation and activation by inflammatory proteins.
Presence of immunological targets

Destruction of virus-infected cells by the adapted immune system requires recognition of these cells. Immunological targets on infected cells are (i) viral proteins expressed on the cell membrane during replication of enveloped viruses and (ii) viral peptides presented together with MHC-I molecules on the cell membrane. Recognition of the first target by antibodies can lead to cell destruction via complement (antibody-dependent complement-mediated lysis - ADCML) or via activated natural killer cells, neutrophils, monocytes or macrophages (antibody-dependent cell-mediated cytotoxicity - ADCC). Recognition of the second target by CD8\(^+\) cytotoxic T-lymphocytes can lead to apoptosis of the infected cell.

In this thesis, it has been shown that, in vitro, half of the FIPV-infected monocytes do not express detectable levels of viral proteins on their surface. This absence of surface-expressed viral proteins can be considered as an immune evasion process since antibodies cannot efficiently recognize the infected cell. Lack of surface expression means that the viral proteins are efficiently retained inside these monocytes. This retention probably occurs in the ER-to-Golgi intermediate compartment (ER-GIC), the budding site of coronaviruses. Both the S and the M proteins contain putative retention motifs. For example, a retention motif (KXHXX) has been described in the cytoplasmic tail of the S protein of FIPV (Lontok et al, 2004). Deletion of this motif leads to more surface-expressed S proteins in transfected cells, in comparison to full length S protein (Dye et al, 2007). The fact that in our experiments half of the monocytes do show surface-expressed viral proteins could be due to higher protein expression levels and therefore saturation of the retrieval machinery.

The FIPV-infected monocytes that do show surface-expressed viral proteins were further studied in our FIPV research group (Dewerchin et al, 2006; 2008; Dewerchin, 2008). A second immune evasion mechanism was detected, namely the antibody-mediated internalization of the surface-expressed viral proteins. Virus-specific antibodies bind to the surface-expressed viral antigens, followed by fast internalization of these antibody-antigen complexes into the monocyte. This mechanism leaves the infected cell cleared from all detectable viral antigens (Dewerchin et al, 2006). During characterization of this mechanism, it was seen that
there was a strong up-regulation of myosin 1 in infected cells (Dewerchin et al, 2008).

These *in vitro* experiments revealed two possible immune evasion strategies at the level of the FIPV-infected monocyte. A logical question was whether these *in vitro* results correlated with the *in vivo* situation. Thus, whether infected cells present in a cat with FIP showed surface-expressed viral proteins. In the second part of this thesis, FIPV-infected cells were successfully isolated from granulomas and exudates from 12 naturally infected cats and *ex vivo* examined. No viral proteins were detected on the surface of FIPV positive cells. It was not examined which mechanism lied behind this absence of surface-expressed viral antigens. However, the results of the cultivation experiment demonstrated that about half of the infected cells cannot express viral antigens on their surface. This indicated that the viral proteins were retained inside the cell. This observation was consistent with the *in vitro* findings found in this thesis and described above.

It has not yet proven if the antibody-mediated internalization of surface-expressed viral proteins, seen *in vitro*, is also responsible for the absence of viral antigens on the surface of *ex vivo* isolated FIPV positive cells (which are capable of expressing viral antigens). There are two indications that the infected cells act similarly *in vitro* and *in vivo*: (i) detection of antibodies in the cytoplasm of infected cells isolated from a cat with FIP (ii) up-regulation of myosin 1 in infected cells isolated from a cat with FIP (unpublished data). Further *ex vivo* co-localization studies of antibodies, viral antigens and myosin in the cytoplasm of FIPV-positive cells are in progress in our FIP research group to support this theory.

The presence of another immunological target, MHC-I molecules, was studied on the infected cells isolated from cats with FIP. On all FIPV positive cells MHC-I was present, showing that no full internalization or retention of the MHC-I molecules occurs. Whether the CD8\(^+\) cytotoxic T-lymphocytes could recognize and destroy the infected cell is not known, since other factors are important e.g. the up-loading of viral peptides on the MHC-I molecules or the presence of down-regulation of MHC-I molecules. Down-regulation has been described for other coronaviruses namely SARS-CoV and mouse hepatitis virus (MHV) (Redwine et al, 2001; Cameron et al, 2008). It has been described for pseudorabies virus (PRV), a herpesvirus, that MHC-I molecules can co-internalize with the antibody-mediated internalized surface-
expressed viral proteins (Favoreel et al, 1999). Since in half of the FIPV-infected cell this antibody-mediated internalization of surface-expressed viral proteins also occurs, it would be interesting to determine \textit{in vitro} if MHC-I molecules also co-internalize, which could give a MHC-I down-regulation.

\textbf{Immune evasion mechanism}

In the last part of this thesis, it was studied whether the clearance of all detectable viral proteins from the plasma membrane protects these cells from immune mediated killing. One mechanism was tested \textit{in vitro}, namely the antibody-dependent, complement-mediated lysis (ADCML). Absence of viral proteins in the plasma membrane of infected monocytes can protect infected cells from efficient ADCML. This has been described for pseudorabies virus (PRV), equine herpesvirus type 1 (EHV 1) and porcine reproductive and respiratory syndrome virus (PRRSV) (van der Meulen et al, 2003; Van de Walle et al, 2003; Costers et al, 2006). It was also tested whether there was efficient ADCML of FIPV-infected monocytes and CrFK cells that show surface-expressed viral antigens.

As expected, no ADCML was detected in monocytes without surface-expressed viral antigens due to lack of antibody binding to the infected cells. Surprisingly, lysis was neither observed in the CrFK cells and the monocytes that do show surface-expressed viral proteins. The amount of bound antibodies is important for the effectiveness of the ADCML (Joseph et al, 1976). All FIPV-infected CrFK cells showed high amounts of bound antibodies on their surface with formation of patches. Patching is not likely to have an influence on the ADCML assay, as has been described for measles virus infected cells (Perrin et al, 1976). The possibility that insufficient amounts of antibodies were bound on infected CrFK cells and monocytes to have a detectable effect of ADCML is unlikely, but cannot be completely excluded. Higher concentrations of complement can also enhance the effectiveness of ADCML (van der Meulen et al, 2003). All the ADCML-assays described in this thesis were performed with 5% complement, but increasing the complement concentration to 10% had no effect on the outcome of the ADCML-assays (unpublished data).

It seems that feline infectious peritonitis virus can employ another immune evasion strategy against ADCML (besides preventing surface expression): the inhibition of
Complement mediated lysis of infected cells. The new immune evasion strategy has to be attributed to the structural proteins or the non-structural proteins of ORF 1ab and not to the accessory or group specific proteins 3abc and 7ab since lysis of cells infected with the double mutant strain FIPV \( \Delta \)3abc/\( \Delta \)7ab was also not detected. It is possible that FIPV employs a new mechanism responsible for this evasion strategy, but it can also show similarities with other viruses. There are three mechanisms described: (i) expressing of proteins with Fc receptor activity that can inhibit the binding of complement to the antibody-antigen complex, (ii) encoding for proteins with functional similarities to complement control proteins which inhibits the complement cascade and (iii) inducing an up-regulation of host complement control factors in the infected cell. For FIPV, the presence of an Fc receptor activity has never been studied but the spike proteins of other coronaviruses, namely mouse hepatitis virus (MHV), bovine coronavirus (BCoV) and transmissible gastroenteritis virus (TGEV) do display Fc receptor activity (Oleszak et al, 1995). That Fc-receptor activity protects against ADCML has been described for herpes simplex virus (HSV) and PRV (Adler et al, 1978; Lubinski et al, 2002; Van de Walle et al, 2003). No viral complement control proteins or up-regulation of host complement control proteins have been described for coronaviruses. Several viral complement control proteins have been described for poxviruses and herpesviruses e.g. viral proteins that bind to or accelerate decay of certain complement factors (reviewed by Favoreel et al, 2003; Bernet et al, 2003). Up-regulation of host complement control proteins has for example been described for human cytomegalovirus (HCMV) where up-regulation of complement regulator CD55 protected infected cells from complement-mediated lysis (Spiller et al, 1996).

We hypothesized in the past that blocking of the antibody-mediated internalization of viral proteins would result in infected cells being recognized and destroyed by the immune system. Therefore, this could be a part of a treatment protocol for FIP cats. Unfortunately, the results in this thesis showed that even if there are viral proteins present on the plasma membrane, no lysis will occur through ADCML. However, if antibodies bind to the surface-expressed viral proteins, cell lysis may still occur via other cell lysis mechanisms e.g. ADCC. The effectiveness of these mechanisms will be studied in the near future in our FIP research group.
The infected cell in the FIPV pathogenesis

In conclusion, the following model summarizes the (possible) immune evasion mechanism present during FIPV pathogenesis which allows infected cells to spread in the presence of antibodies. This model is schematically represented in Figure 6.1. The main target cells of the FIPV are the monocyte/macrophages. At the virus-monocyte level, an immune evasion mechanism has been described by other research groups, namely the antibody-dependent enhancement of infectivity (ADEI) (Figure 6.1A). Antibodies, at a low concentration, bind to free virus and facilitate and enhance in vitro infection of monocytes via Fc receptor mediated up-take (Hohdatsu et al, 1991; Corapi et al, 1992; Olsen et al, 1992, 1993; Takano et al, 2008). This suggests that in the presence of antibodies a larger number of cells may be infected resulting in a faster spread of the virus. ADEI is also proposed as the reason why a more fulminating disease progression of FIP is seen in experimentally inoculated seropositive animals (Pedersen and Boyle, 1980; Weiss and Scott, 1981a,b; Pedersen and Black, 1983; Vennema et al, 1990). However, in natural infections of seropositive animals no enhancement of infectivity has been reported (Addie et al, 1995). Also, the high antibody concentrations present in a cat with FIP neutralize the virus in vitro in stead of giving ADEI. But it is possible that in the cat low antibody concentrations are present locally, e.g. in the granulomas, to allow ADEI.

FIPV replicates highly effective in the monocytes. Half of the infected monocytes remain invisible to the humoral immune system, since no viral antigens are present on their plasma membrane (Figure 6.1B). This protects the cell against ADCML and most probably also against ADCC. The other half of the cells do express viral antigens on their plasma membrane. Binding of antibodies to these surface-expressed viral antigens lead to internalization of these antibody-antigen complexes leaving the cell again invisible for the humoral immune system (Figure 6.1E). Additionally, even if the immune system would have the time or the possibility to attack the infected cell, the ADCML of FIPV-infected cell is also inhibited by a virus-induced immune evasion mechanism (Figure 6.1D). MHC-I molecules are present on these infected cells, but this is no guarantee that the lysis of these cells by cytotoxic T-lymphocytes is efficient.
Thus, FIPV and FIPV-infected cells exhibit several immune evasion mechanisms to avoid clearance by the humoral immune system. This correlates with the general belief that the cell-mediated immunity plays a decisive role in the control of FIP. A strong cell-mediated immune response seems protective against FIP. However, T-lymphocyte depletion occurs early in infection and correlates with enhanced viral replication (Haagmans et al, 1996; de Groot-Mijnes et al, 2005). It still has to be proven if this depletion is an immune evasion mechanism by inhibiting the attack of the cytotoxic T-lymphocytes (Figure 6.1C). It appears that FIPV has become a master in disguise, showing several (possible) immune evasion mechanisms against both humoral and cell-mediated immune responses.

**Future goals and open questions**

The future goals of our FIPV research are to study the immune mechanisms that lead to destruction of virus-infected cells, to search for immune evasion mechanisms and to search for the viral proteins responsible for these mechanisms. Several research lines can be set out.

(i) The effectiveness of the destruction of FIPV-infected cells through ADCC.
(ii) The effectiveness of the destruction of FIPV-infected cells by cytotoxic T-lymphocytes.
(iii) Clarifying which viral complement evasion strategy FIPV uses to inhibit ADCML.

The ultimate goal of these experiments is to develop a treatment protocol that is aimed at inhibiting the immune evasion strategies to give the cat’s immune system the chance to recognize and destroy the virus-infected cell and overcome the infection. Depending on the result of the experiments, this treatment could be aimed at inhibiting the internalization process and at targeting the viral proteins that are responsible for the complement evasion process and inhibit their immune evasion function.

A different approach to tackle the problem of FIP could be focusing on the resistant cats. Demonstrating a genetic correlation between *in vivo* and *in vitro* resistance against FCoV could lead to obtaining an increased resistance against FCoV through selective breeding.
Figure 6.1: Possible immune evasion mechanisms present at the level of FIPV-infected cells: (A) FIPV up-take can be facilitated by antibodies e.g. antibody dependent enhancement of infectivity (ADEI), (B) intracellular retention of viral proteins and (E) internalization of surface-expressed viral proteins inhibits the recognition of FIPV-infected cells by antibodies, which prevents the antibody-dependent, complement-mediated lysis (ADCML) and the antibody-dependent cell-mediated cytotoxicity (ADCC), (D) the ADCML is inhibited even if antibodies are able to recognize the cell and (C) a T-lymphocyte depletion is seen during FIPV-infection.
References


Costers S, Delputte P, Nauwynck H (2006) Porcine reproductive and respiratory syndrome virus-infected alveolar macrophages contain no detectable levels of viral proteins in their plasma membrane and are protected against antibody-dependent, complement-mediated cell lysis. J Gen Virol 87(8), 2341-2351


Summary / Samenvatting
Feline coronaviruses (FCoV) are common pathogens of cats. Two biotypes have been described: the endemic feline enteric coronavirus (FECV) and the less common feline infectious peritonitis virus (FIPV). During FECV infection, the virus can disseminate throughout the body but only causes a mild and mostly asymptomatic enteritis. Occasionally FECV mutates to FIPV which causes a highly fatal systemic infection with fibrinous-granulomatous serositis. The pathogenesis of FIP and the relationship between the different FCoV biotypes has partly been clarified but this has not led to effective treatment or prevention. More aspects of the pathogenesis have yet to be elucidated. Especially the interaction of the viruses and the virus-infected cell with the immune system needs to be examined, since it is not clear why in most cases the cat’s immune system can not overcome a FIPV infection.

The aims of this doctoral research were (i) examining the replication of FCoV in the blood monocyte to determine if there was a difference between FIPVs and FECVs replication \textit{in vitro} that could explain the differences \textit{in vivo} (ii) examining the presence of immunological targets (surface-expressed viral antigens and MHC-I molecules) on \textit{in vitro} infected cells and on infected cells isolated from cats with FIP (\textit{ex vivo}). This to determine if immune evasion processes on the level of infected cells were present and if these processes could protect the cell against antibody-dependent, complement-mediated lysis.

In \textbf{Chapter 1}, an introduction is given on the FCoV that focuses on classification, virus structure, host range, epidemiology, pathogenesis, clinical signs, diagnosis, treatment and prevention. Additionally, a limited but general overview of the (viral) immune response and immune evasion is given. This is followed by the known immune responses and immune evasion mechanisms seen with FCoV.

In \textbf{Chapter 2}, an outlining of the aims is given.
In Chapter 3, infection kinetics of FIPV (strain 79-1146) and FECV (strain 79-1683) were performed in peripheral blood monocytes of 22 cats and compared to those in Crandell feline kidney (CrFK) cells, a continuous cell line often used in FCoV research. Both virus titers and antigen expression were examined. Surprisingly, the infection kinetics differed depending on the cat from which the monocytes were isolated. Three different infection patterns were detected. In the first two patterns both FECV and FIPV could initially infect the monocytes, but in the first pattern only the FIPV infection was progressive while the FECV infection was not sustained. In the second pattern both FIPV and FECV infection was not sustained. In the third pattern the monocytes were not susceptible to either FECV or FIPV infection. The FIPV and FECV infection rate in monocytes was lower than 1%. Overall it was seen that the FECV infection was never sustained and that the total virus production (viral load) was up to 100 times lower in FECV-infected monocytes compared to FIPV-infected monocytes. This difference in lack of sustainability and long-term production could explain why the two biotypes act so differently in vivo. Both FIPV and FECV could infect the monocytes in vitro, indicating that both viruses can reach the blood stream. The hypothesis is that only systemic effect and clinical signs are seen with FIPV because of the higher viral load and progressive infection in the monocyte.

Studying the surface-expressed viral antigens on infected monocytes showed that about half of the FIPV- and FECV-infected monocytes expressed viral proteins in their plasma membrane. The other half of the infected cells efficiently retained the proteins inside the cell. This retention can be an immune evasion mechanism since antibodies can not recognize the infected cell anymore.

In CrFK cells, no difference was observed between FECV and FIPV replication. This replication was also very different from those in monocytes: up to 90% of the CrFK cells could be infected, fewer infectious viruses were produced per cell and almost all infected cells showed surface-expressed viral proteins. This indicates that the CrFK cell line is not suited for studying FCoV-host cell interactions.

In Chapter 4, virus-infected cells were isolated from exudates and/or granulomas of naturally FIPV-infected cats. Using immunofluorescence stainings different characteristics were determined: (i) if these cells belonged to the
monocyte/macrophage lineage, (ii) their viability and (iii) the presence of surface-expressed viral antigens and MHC-I molecules. Of the FIPV-positive cells, $95 \pm 5\%$ belonged to the monocyte/macrophage lineage. On these FIPV-positive cells, no surface-expressed viral antigens were detected while MHC-I molecules were present. These cells were cultivated in vitro, to determine whether they were capable of expressing viral proteins on their surface. Half of the FIPV-positive cells ($52 \pm 10\%$) re-expressed viral proteins in their plasma membrane. This indicates that about half of the infected cells can not express viral antigens on their surface which is consistent with the in vitro findings. Which mechanism lies behind the in vivo absence of surface-expressed viral antigens on infected cells capable of expressing viral proteins is not yet known. An internalization process, described by our FIPV research group, in which in vitro binding of antibodies to the surface-expressed FIPV proteins led to internalization of all antibody-protein complexes, could be the cause.

The presence of MHC-I molecules proves that no full internalization or retention of these molecules occurs. But with the used techniques it was not possible to quantify the number of MHC-I molecules or to determine whether they were loaded with FIPV peptides.

In Chapter 5, it was studied in vitro whether the absence of viral proteins in the plasma membrane protected FIPV (strain 79-1146) infected cells from antibody-dependent, complement-mediated lysis (ADCML). It was also tested if there was efficient ADCML of FIPV-infected cells that do show surface-expressed viral antigens. ADCML assays were performed on (i) infected CrFK cells that show surface-expressed viral antigens since they are not capable of internalizing antibody-antigen complexes (ii) infected monocytes without surface-expressed viral proteins due to retention and (iii) infected monocytes with surface-expressed viral proteins due to blockage of the antibody-mediated internalization by an inhibitor.

As expected, no ADCML of the monocytes without surface-expressed viral antigens was detected. Surprisingly, no lysis was observed in the CrFK cells and the monocytes that do show surface-expressed viral proteins. Controls with pseudorabies virus-infected CrFK cells and monocytes showed that the ADCML-assays were functional though.
These experiments prove that FIPV can employ another immune evasion strategy: the inhibition of ADCML of infected cells that show surface-expressed viral antigens. This new evasion strategy is not attributed to the group specific proteins 3abc and 7ab since lysis of cells infected with the double mutant strain FIPV Δ3abc/Δ7ab was also not detected. The new immune evasion strategy has to be attributed to the structural proteins or the non-structural proteins of ORF 1ab and can occur directly via viral proteins or indirectly by means of cellular proteins that regulate the complement cascade.

**Chapter 6** gives a general discussion on the results obtained in this thesis. A schematically presentation is given of the immune evasion mechanisms on the level of the FIPV-infected cell with the ones found during this doctoral research and the ones already described. Goals of our FIPV research group are presented.

Several important conclusions can be drawn from this thesis:

(i) Both FECV and FIPV can infect monocytes. FECV infections show a lack of sustainability and long-term production in comparison to FIPV infection. This could explain the differences between FIPV and FECV pathogenesis.

(ii) Half of the FIPV-infected monocytes do not express viral antigens on their surface. This is an immune evasion mechanism since it protects these infected cells against ADCML.

(iii) Infected cells present in naturally FIPV infected cats do not express viral antigens on their surface. This absence seems consistent with the *in vitro* findings of our FIPV research group.

(iv) FIPV can inhibit the ADCML of infected cells that show surface-expressed viral antigens. This newly found immune evasion mechanism is attributed to the structural proteins or the non-structural proteins of ORF 1.
Samenvatting

Felien coronavirussen (FCoV) zijn vaak voorkomende pathogenen bij katten. Er bestaan twee biotypes: de endemisch voorkomende felien enterisch coronavirus (FECV) en de minder vaak voorkomende felien infectieuze peritonitis virus (FIPV). Tijdens een FECV infectie kan het virus verspreiden in het lichaam, maar het veroorzaakt enkel een milde en meestal subklinische darmontsteking. Nu en dan muteert FECV naar FIPV, dat een zeer dodelijke systemische infectie kan veroorzaken, welke gekenmerkt wordt door een fibrineuze-granulomateuze serositis. De pathogenese van FIP en de relatie tussen de twee verschillende FCoV biotypes is gedeeltelijk gekend, maar dit heeft nog niet geleid tot een doeltreffende behandeling of preventie. Meerdere aspecten van de pathogenese moeten nog opgehelderd worden. Belangrijk hierin is de interactie van de virussen en de virus-geïnfecteerde cel met het immuunsysteem, aangezien het niet duidelijk is waarom het immuunsysteem van de kat in de meeste gevallen niet in staat is om een FIPV infectie te overwinnen.

De doelstellingen van dit doctoraatsonderzoek waren (i) het onderzoeken van de vermeerdering van FCoV in de bloedmonocyten om te bepalen of er een verschil is tussen FIPV en FECV vermeerdering in vitro, wat mogelijk een verklaring zou kunnen geven voor de in vivo verschillen en (ii) het bepalen van de aanwezigheid van immunologische targets (virale proteïnen en MHC-I moleculen) op het plasmamembraan van in vitro geïnfecteerde cellen en van geïnfecteerde cellen geïsoleerd uit katten met FIP (ex vivo). Zo kon bepaald worden of er immunoevasieve processen aanwezig waren op het niveau van de virus-geïnfecteerde cel en of deze processen de geïnfecteerde cel beschermden tegen antistof-afhankelijke, complement-gemedierte lyse.

In Hoofdstuk 1 wordt een inleiding gegeven over het FCoV, met de nadruk op hun classificatie, virusstructuur, epidemiologie, pathogenese, symptomen, diagnose, behandeling en preventie. Daarnaast wordt een algemeen overzicht gegeven over de
virale immuunrespons en de immuno-evasie. Vervolgens worden de gekende immuunreacties en immuno-evasie mechanismen van FCoV besproken.

In **Hoofdstuk 2** worden de doelstellingen van deze thesis uiteengezet.

In **Hoofdstuk 3** werden de infectiekinetieken van FIPV (stam 79-1146) en FECV (stam 79-1683) in perifere bloedmonocyten van 22 katten bepaald. Deze werden vergeleken met de kinetieken in Crandell feline kidney (CrFK) cellen, een continue cellijn die vaak gebruikt wordt in FCoV onderzoek. Zowel virustiters als expressie van virale antigenen werden onderzocht. Het verloop van de infectiekinetieken was afhankelijk van de kat waaruit de monocyten waren geïsoleerd. Drie infectiepatronen werden waargenomen. In de eerste twee patronen kon zowel FECV als FIPV de monocyten initiëel infecteren, maar in het eerste patroon was enkel de FIPV infectie progressief terwijl de FECV infectie niet werd onderhouden (een abortieve infectie). In het tweede patroon vertoonden zowel FIPV als FECV een abortieve infectie. In het derde patroon waren de monocyten ongevoelig voor zowel FECV als FIPV. De FIPV en de FECV infectiegraad voor alle patronen was lager dan 1%. Algemeen werd gezien dat de FECV infectie steeds abortief was en dat de totale virus productie 100 maal lager lag in FECV-geïnfecteerde monocyten in vergelijking met FIPV-geïnfecteerde monocyten. Dit verschil in lange termijn infectie en virusproductie zou kunnen verklaren waarom de twee biotypes zo verschillend zijn *in vivo*. Zowel FECV als FIPV konden initiëel de monocyten infecteren *in vitro*, wat erop wijst dat beide virussen de bloedbaan kunnen bereiken. De hypothese is dat enkel een systemisch effect en klinische symptomen gezien wordt bij FIPV door de hogere virusproductie en de progressieve infectie in de moncyt.

Het bepalen van de aanwezigheid van virale antigenen toonden aan dat in ongeveer de helft van de FIPV- en FECV-geïnfecteerde cellen virale proteïnen tot expressie werden gebracht in hun plasmamembraan. De ander helft van de geïnfecteerde cellen weerhielden deze proteïnen volledig binnenin de cel. Deze intracellulaire retentie is mogelijk een immuno-evasie mechanisme omdat de antistoffen niet meer in staat zijn om de geïnfecteerde cel te herkennen.
In CrFK cellen werd geen verschil gezien tussen de FECV en FIPV infectiekinetieken. Deze kinetieken waren zeer verschillend met deze in de monocyt: (i) tot 90% van de CrFK cellen konden geïnfecteerd geraken, (ii) er werd minder virus geproduceerd per cel en (iii) zo goed als alle geïnfecteerde cellen brachten virale proteïnen tot expressie in hun plasmamembraan. Dit wijst erop dat CrFK cellen niet geschikt zijn om de FCoV-gastheercel interacties te onderzoeken.

In Hoofdstuk 4 werden virus-geïnfecteerde cellen geïsoleerd uit exsudaat en/of granuloma’s van FIPV-geïnfecteerde katten. Door middel van immunofluorescentie-kleuringen werden verschillende eigenschappen onderzocht: (i) of deze cellen behoorden tot de monocyte/macrofaag lijn, (ii) hun viabiliteit en (iii) de aanwezigheid van virale antigenen en MHC-I moleculen op hun plasma membraan. Van de FIPV-positieve cellen behoorden 95 ± 5% tot de monocyte/macrofaag lijn. Op hun plasmamembran werden geen virale antigenen gedetecteerd, maar MHC-I moleculen waren wel aanwezig. Deze cellen werden in vitro gecultiveerd om te bepalen of ze in staat zijn om virale proteïnen tot expressie te brengen in hun plasmamembraan. De helft van de gecultiveerde FIPV-positieve cellen (52 ± 10%) brachten virale proteïnen opnieuw tot expressie. Dit wijst erop dat ongeveer de helft van de in vivo geïnfecteerde cellen niet in staat zijn virale antigenen in hun plasmamembraan tot expressie te brengen, wat overeenkomt met de in vitro resultaten. Welk mechanisme verantwoordelijk is voor de in vivo afwezigheid van virale antigenen op het plasmamembran van geïnfecteerde cellen die wel in staat zijn tot expressie van deze proteïnen, is niet gekend. Een internalisatie proces, beschreven door onze FIPV onderzoeksgroep, waarin in vitro binding van antistoffen op plasmamembraan-geassocieerde FIPV proteïnen leidt tot internalisatie van alle antistof-proteïne complexen, kan de oorzaak zijn.

De aanwezigheid van MHC-I moleculen toonde aan dat er geen volledige internalisatie of retentie van deze moleculen gebeurde. Met de gebruikte technieken was het echter niet mogelijk om hun aantal te kwantificeren of te bepalen of de MHC-I moleculen FIPV peptiden presenteerden.

In Hoofdstuk 5 werd in vitro onderzocht of de afwezigheid van virale proteïnen in de plasmamembraan van FIPV (stam 79-1146) geïnfecteerde cellen bescherming
bood tegen antistof-afhankelijke, complement-gemedieerde lyse (ADCML). Er werd ook getest of er een efficiënte ADCML was van FIPV-geïnfecteerde cellen die wel virale proteïnen tot expressie brachten in hun plasmamembraan. ADCML-testen werden uitgevoerd op (i) geïnfecteerde CrFK cellen die virale proteïnen tot expressie brengen in hun plasmamembraan, aangezien ze niet in staat zijn om antistof-antigen complexen te internaliseren, (ii) geïnfecteerd monocyten zonder virale proteïnen in hun plasmamembraan door retentie en (iii) geïnfecteerde monocyten met virale proteïnen in hun plasmamembraan doordat de antistof-gemedieerde internalisatie werd geblokkeerd door een inhibitor. Zoals verwacht, werd er geen ADCML gedetecteerd van de monocyten zonder virale proteïnen in hun plasmamembraan. Zeer verrassend, werd er echter ook geen lyse opgemerkt van zowel de CrFK cellen als de monocyten die wel virale proteïnen tot expressie brachten in hun plasmamembraan. Controles met pseudorabiës virus geïnfecteerde CrFK cellen en monocyten bevestigden dat de ADCML-testen functioneel waren. Deze experimenten toonden aan dat FIPV nog een ander immuno-evasie mechanisme kan gebruiken: de inhibitie van ADCML van geïnfecteerde cellen met plasmamembranaan-geassocieerde virale proteïnen. Deze nieuwe evasie strategie kan niet toegeschreven worden aan de accessoire of groepspecifieke proteïnen 3abc en 7ab aangezien er ook geen lyse werd opgemerkt van cellen geïnfecteerd met de dubbele mutante stam FIPV Δ3abc/Δ7ab. Dit wijst erop dat de nieuwe immuno-evasie strategie toegeschreven moet worden aan de structurele proteïnen of de niet-structurele proteïnen van ORF 1ab. Deze strategie kan direct veroorzaakt worden door de virale proteïnen of indirect via cellulaire proteïnen die de complement cascade reguleren.

In **Hoofdstuk 6** werd een algemene discussie gegeven over de resultaten verkregen in deze thesis. Een schematische voorstelling werd gegeven van de immuno-evasie mechanismen op het niveau van de FIPV-geïnfecteerde cel met zowel diegenen gevonden tijdens dit doctoraatsonderzoek als diegenen die reeds beschreven waren.
Verscheidene belangrijke conclusies kunnen uit deze thesis getrokken worden:

(i) Zowel FECV als FIPV kunnen monocyten infecteren. FECV infecties vertoonden een gebrek aan lange termijn infectie en lagere virusproductie in vergelijking met FIPV infecties. Dit verschil zou een verklaring kunnen zijn voor het verschil in FIPV en FECV pathogenese.

(ii) De helft van de FIPV-geïnfecteerde monocyten vertoonden geen expressie van virale proteïnen in hun plasmamembraan. Deze afwezigheid is een immuno-evasie mechanisme aangezien het deze geïnfecteerde cellen beschermt tegen ADCML.

(iii) Geïnfecteerde cellen, aanwezig in natuurlijke FIPV-geïnfecteerde katten, vertonen geen expressie van virale antigenen in hun plasmamembraan. Deze afwezigheid komt sterk overeen met de in vitro bevindingen beschreven door onze FIPV onderzoeksgroep.

(iv) FIPV kan de ADCML inhiberen van geïnfecteerde cellen met plasmamembraan-geassocieerde virale proteïnen. Dit nieuwe immuno-evasie mechanisme kan toegeschreven worden aan de structurele proteïnen of de niet-structurele proteïnen van ORF 1.
Curriculum vitae

Personalia

Publicaties

Publicaties in internationale wetenschappelijke tijdschriften.


Abstracts

Cornelissen E, Dewerchin H, Nauwynck H. Replication of feline coronaviruses in peripheral blood monocytes. International congress of veterinary virology: comparative and emerging virus infections of dogs and cats - European Society of Veterinary Virology (ESVV), 20\textsuperscript{th}-22\textsuperscript{nd} June 2005, Liverpool, United Kingdom.

Dewerchin H, Cornelissen E, Nauwynck H. Internalization of plasma membrane-bound viral proteins upon antibody addition in feline infectious peritonitis virus infected monocytes occurs through a novel internalization pathway, a putative immune-evasion proves. X\textsuperscript{th} International Nidovirus Symposium: Toward Control of SARS and other Nidovirus Diseases, 25\textsuperscript{th}-30\textsuperscript{th} June 2005, Colorado Springs, Colorado, United States of America.

Dewerchin H & Cornelissen E, Van Hamme E, Nauwynck H. Possible immune evasion strategies of feline infectious peritonitis virus. Belgian Society for Microbiology: 12\textsuperscript{th} annual symposium, 24\textsuperscript{th} November 2006, Brussels, Belgium.

Cornelissen E, Dewerchin H, Van Hamme E, Nauwynck H. Absence of surface expression of feline infectious peritonitis virus (FIPV) antigens on infected cells isolated from cats with FIP. 7th International Congress of veterinary virology - ESVV, 24\textsuperscript{th}-27\textsuperscript{th} September 2006, Lisbon, Portugal.

Van Hamme E, Dewerchin H, Cornelissen E, Nauwynck H. Attachment and internalization of feline infectious peritonitis virus in monocytes and Crandell feline kidney cells.

- Early steps of virus life cycle: molecular and cellular insights. 4\textsuperscript{th}-5\textsuperscript{th} October 2007, Institut Pasteur, Paris, France.
- Belgian Society for Microbiology: 13\textsuperscript{th} annual symposium, 23\textsuperscript{th} November 2007, Brussels, Belgium.
- XIth international symposium on nidoviruses. 22nd-27th June 2008, Oxford, United Kingdom.
- Belgian Society for Microbiology: 14th annual symposium, 12th December 2008, Brussels, Belgium.

- XIth international symposium on nidoviruses. 22nd-27th June 2008, Oxford, United Kingdom.
- Belgian Society for Microbiology: 14th annual symposium, 12th December 2008, Brussels, Belgium.

- XIth international symposium on nidoviruses. 22nd-27th June 2008, Oxford, United Kingdom.
- Belgian Society for Microbiology: 14th annual symposium, 12th December 2008, Brussels, Belgium.
**Mondelinge presentaties**


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