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Short communication

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

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Abstract

Feline infectious peritonitis virus (FIPV) positive cells are present in pyogranulomas and exudates from cats with FIP. These cells belong mainly to the monocyte/macrophage lineage. How these cells survive in immune cats is not known. In this study, FIPV positive cells were isolated from pyogranulomas and exudates of 12 naturally FIPV-infected cats and the presence of two immunologic targets, viral antigens and MHC I, on their surface was determined. The majority of the infected cells were confirmed to be cells from the monocyte/macrophage lineage. No surface expression of viral antigens was detected on FIPV positive cells. MHC I molecules were present on all the FIPV positive cells. After cultivation of the isolated infected cells, $52 \pm 10\%$ of the infected cells re-expressed viral antigens on the plasma membrane.

In conclusion, it can be stated that in FIP cats, FIPV replicates in cells of the monocyte/macrophage lineage without carrying viral antigens in their plasma membrane, which could allow them to escape from antibody-dependent cell lysis.

Keywords: Feline infectious peritonitis; Viral antigen-expression; MHC I; Immune-evasion

1. 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 serosae 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 pyogranulomas and, based on morphology and the granulocyte/monocyte/macrophage marker calprotectin, defined as macrophages (Weiss and Scott, 1981; Kipar et al., 1998). Infected mononuclear cells were also isolated from exudates (Cammarata Parodi et al., 1993; Paltrinieri et al., 1999). In one way or another, these infected cells succeed in staying alive and transmitting

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virus to new susceptible cells in the presence of a high concentration of antibodies. How they do this, is not known. 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 and Oldstone, 1980). For pseudorabies virus (PRV) end equine herpesvirus-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, 2006; Van de Walle et al., 2003).

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 et al., 2005, 2006).

Besides through antibody-mediated lysis, the adaptive immune system can eliminate virus-infected cells through cell-mediated immunity. Some of the newly synthesized viral proteins in infected cells are disintegrated by proteasomes, the peptides are coupled to major histocompatibility complex I (MHC I) 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, it was examined in infected monocytes/macrophages of FIP cats if viral antigens are expressed on the plasma membrane and if the expression of MHC I molecules was inhibited.

2. Material and methods

2.1. 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 titre and type of FIP (effusive or non-effusive) are listed in Table 1.

Table 1
Breed, age, sex, FCoV antibody titre and pathological form of FIP from the cats enclosed in this study

Cat no.	Breed	Age (months)	Sex	FCoV antibody titre[a]		Pathological form
				Serum	Exudate	
1	British shorthair	10	Male	>12800	–	Non-effusive
2	Bengale	9	Male	1280	3200	Effusive
3	Persian	9	Female	1280	1280	Effusive
4	Persian	4	Male	1600	1280	Effusive
5	Persian	36	Male	6400	6400	Effusive
6	Exotic shorthair	5	Female	640	320	Effusive
7	British shorthair	9	Female	6400	1280	Effusive
8	Sphynx	7	Male	>12800	>12800	Effusive
9	Sphynx	4	Male	1280	–	Non-effusive
10	Sphynx	2	Female	>12800	3200	Effusive
11	Persian	42	Female	>12800	3200	Effusive
12	Sphynx	6	Female	6400	–	Non-effusive

–: Non-effusive form of FIP, exudate not present.
[a] IPMA antibody titre.

2.2. Antibodies

Monospecific, polyclonal antibodies originating from cats infected with serotype II FIPV 79-1146 were kindly provided by Dr. Egberinck (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 serotypes 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 the 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 serotypes 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).

2.3. 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 min at 4 °C. Afterwards, tissues with pyogranulomas were collected. Small blocks containing almost just the pyogranulomas 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 two needles. The cell suspension was then centrifuged at 400 × *g* for 10 min at 4 °C.

2.4. Characterization of FIPV positive cells

The obtained cell suspensions from the exudates and the tissues with pyogranulomas were each divided in three parts on which different stainings in suspension were performed. The cells from the pyogranulomas 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 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 2.

After staining, cells were mounted on microscope slides using glycerin–PBS solution (0.9/0.1, v/v) with 2.5% 1,4-diazabicyclo(2,2,2)octane (Janssen Chimica, Beerse, Belgium) and analyzed by a Leica DM RBE fluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany).

2.5. 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 h. 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.

2.6. Statistics

Results were analyzed with the Wilcoxon signed ranks test. Statistical analyses were performed with SPSS 11.0 (SPSS Inc., Chicago, Illinois, USA).

3. Results

FIPV positive cells were found in cell suspensions from exudates and pyogranulomas in all cats. The percentage of FIPV positive cells varied from <1 to 10% (Table 3). The majority of the FIPV positive cells

Table 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 FIPV positive cells

Different staining steps (1–6)	Staining 1	Staining 2	Staining 3
1. Viability staining	EMA[a,b]	–	EMA[a,b]
2. Fixation	Formaldehyde 1%	Formaldehyde 1%	Formaldehyde 1%
3. Cell surface staining	Monocyte marker (DH59B) + goat anti-mouse-FITC[b]	Biotinylated anti-FIPV Ab + streptavidin–Texas Red[b]	MHC I marker (CF298A) + goat anti-mouse-FITC[b]
4. Permeabilization	Triton X-100[c] 0.1%	Triton X-100[c] 0.1%	Triton X-100[c] 0.1%
5. Cytoplasmic staining	Biotinylated anti-FIPV Ab + streptavidin–Alexa fluor® 350[b] (blue)	Anti-FIPV-FITC Ab	Biotinylated anti-FIPV Ab + streptavidin–Alexa fluor® 350[b] (blue)
6. DNA staining	–	Hoechst 33342[b]	–

[a] Ethidium mono-azide bromide which specifically stains the nuclei of dead cells (red).
[b] Molecular Probes (Eugene, Oregon, USA).
[c] Sigma–Aldrich GmbH (Steinheim, Germany).

($95 \pm 5\%$) belonged to the monocyte/macrophage lineage (mononuclear and DH59B positive) (Fig. 1, lane A). Less than 1% of the FIPV positive cells showed a polymorphonuclear nucleus.

Staining 2 revealed that no infected cells showed expression of viral antigens on their surface (Fig. 1, lane B). After cultivation of the FIPV positive cells, viral antigens were re-expressed on the plasma membrane as soon as 2 h post-seeding. Re-expression only occurred in $52 \pm 10\%$ of infected cells (Fig. 2). 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 (Fig. 1, lane C). No difference in amount

Table 3
Quantification, identification and determination of viability of FIPV positive cells isolated from pyogranulomas and exudates of 9 FIP cats

Cat no.	Origin of the cells	FIPV positive cells[a] (% of total cells)	Monocytes/macrophages (% of FIPV positive cells)	Dead cells (%)	
				FIPV positive monocytes	FIPV negative monocytes
1	Pyogranulomas	5	98	51	38
2	Pyogranulomas + exudates	10	96	18	26
3	Pyogranulomas	1	92	2	4
	Exudate	1	90	10	0
4	Pyogranulomas	ND	ND	ND	ND
	Exudate	<1[b]	86	0	2
5	Pyogranulomas	1	100	38	12
	Exudate	1	100	8	6
6	Pyogranulomas	1	98	21	15
	Exudate	<1[b]	98	12	21
7	Pyogranulomas	1	86	64	14
	Exudate	1	90	2	3
8	Pyogranulomas	<1[b]	100	0	6
	Exudate	1	100	0	0
9	Pyogranulomas	<1[b]	100	7	1

ND: not detected, no antigen positive cells on the slide.
[a] Mononuclear and DH59B positive.
[b] Antigen positive cells present but only a few per slide.

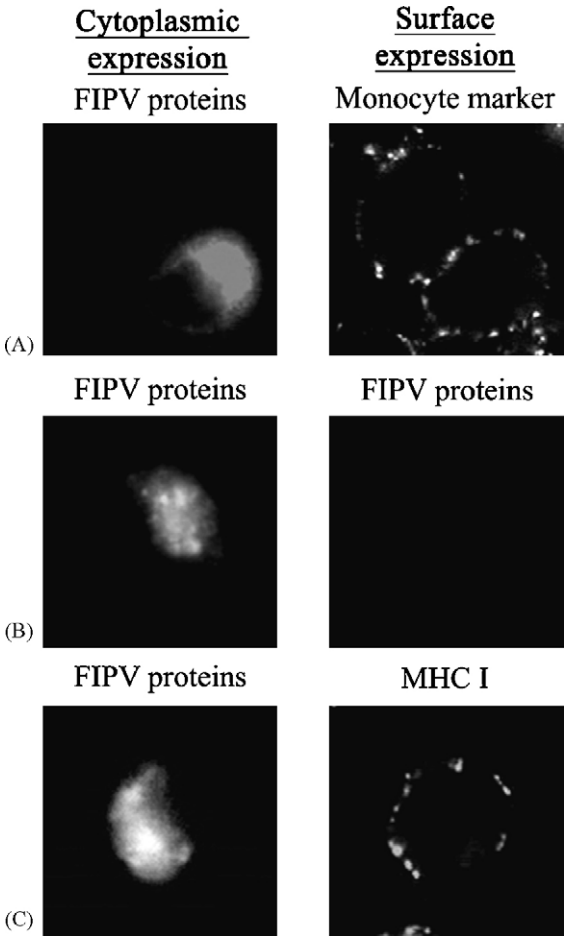

Fig. 1. Surface expression of the monocyte marker (A); absence of surface expression of viral proteins (B); surface expression of MHC I (C) on cytoplasmic FIPV positive cells.

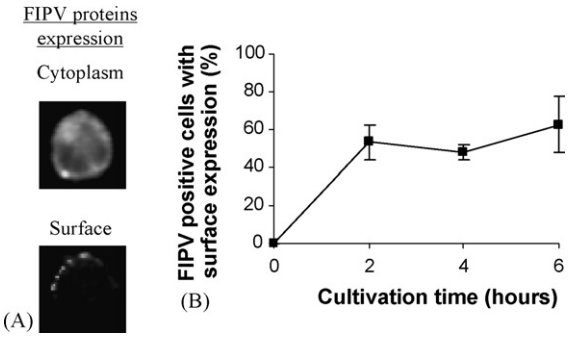

Fig. 2. Re-expression of viral proteins on the plasma membrane after cultivation of FIPV positive cells isolated from three cats with naturally occurring FIP: immunofluorescence pictures (A); kinetics (B).

of MHC I expression was observed between live and dead cells.

The results of the viability staining (staining 1) are given in Table 3. This staining showed that the percentage of dead FIPV positive monocytes in pyogranulomas was significantly higher than the control cells (FIPV negative, DH95B positive cells) ($p < 0.1$), whereas no difference was observed in exudates between FIPV positive monocytes and the control cells.

4. Discussion

In this study, it was shown that FIPV positive monocytes in cats with FIP express MHC I on their surface, but not viral antigens.

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 nine cats with FIP. This could implicate 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 are not capable of expressing viral antigens on their surface. This observation is consistent with the *in vitro* findings of Dewerchin 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 FIPV positive cells isolated from FIP cats that seem to be 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 that do show

surface expressed viral antigens, it most likely occurs also *in vivo* (Dewerchin et al., 2006).

The fact that viral antigens could not be demonstrated with the used staining is not the result of antibodies present in the cat that already bound to these antigens and hinder binding of other antibodies. This hindering is not likely to occur since in FIPV-infected Crandell feline kidney cells, surface expressed FIPV antigens covered by strain specific antibodies were still accessible for other antibodies (data not shown).

Besides 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.

With the exception of two cats, higher cell death in infected cells seems to be present in pyogranulomas. In exudates, this observation was not made. The cause of this difference between pyogranulomas and exudates is not clear. The increased cell death may be due to infection or to the response of the immune system on the infected cell. 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 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 and Black, 1983; Hayashi et al., 1982; Weiss and 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 et al., 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 *in vivo* which may make them invisible for the humoral immune response. In contrast, MHC I molecules are abundantly present on their surface.

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