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Production of IFN-γ in feline whole blood after incubation with potential T-cell epitopes of the nucleocapsid protein of feline coronavirus

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ABSTRACT

Interferon gamma (IFN-γ) plays an important role in cell mediated responses against mutated feline coronavirus strains (FCoV) involved in the pathogenesis of feline infectious peritonitis (FIP). The aim of this study was to establish a combined *in silico* and *in vitro* approach to assess feline leukocyte production of IFN-γ in response to selected peptides of the nucleocapside protein (N) of FCoVs.

To this aim, we designed, through a bioinformatic approach, 8 potentially immunogenic peptides from the protein N corresponding to sequences of residues 14, 182, 198 detected only in FCoVs from FIP cats (virulent strains), only in FCoVs from healthy cats (avirulent strains) and both in FIP and in healthy cats (mixed strains).

The peptides or a sham solution were incubated with whole blood from 16 cats (7 healthy and 9 with chronic diseases other than FIP) and IFN-γ concentration was measured on plasma using an ELISA system. RT-PCR expression of IFN-γ mRNA was also evaluated after incubation of the peptides or a sham solution with whole blood from 4 clinically healthy cats.

The mean plasma concentration of IFN-γ in samples incubated with peptides decreased and the expression of IFN-γmRNA did not change compared with the sham solution, except for some cats with chronic diseases (which probably have a "pre-activated" immune response). These cats responded to "avirulent" or "mixed" peptides by increasing the concentration of IFN-γ and the expression of IFN-γ mRNA.

The combined approach employed in this study allowed us to identify potentially immunogenic peptides of FCoV N protein that can modulate the production of IFN-γ especially in cats with a "pre-activated" cell mediated response.

1. Introduction

Feline infectious peritonitis (FIP) is a fatal immune-mediated disease triggered by infection with feline coronavirus (FCoV). Feline infectious peritonitis virus (FIPV) is a virulent biotype of feline enteric coronavirus (FECV), a low pathogenic FCoV that induces mild enteritis in kittens. The analysis of FIPV and FECV genomes suggests that FIPV may arise from FECV by a series of genomic mutations that provide the virus with the ability to replicate within cells of the monocytic lineage and thus to disseminate throughout the body (Vennema et al., 1998; Pedersen et al., 2009; Chang et al., 2010).

The pathogenesis of FIP is not completely understood, but the development of viral quasispecies and feline immune response to infection are considered to be key pathogenic events. Usually a predominantly humoral immunity response is considered ineffective in virus neutralization since the presence of antibodies could enhance the uptake of FCoVs by macrophages (Pedersen,

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2009). In this type of immune response the deposition of macrophages and virus-antibody complexes around small venules leads to a pyogranulomatous vasculitis that is responsible for the formation of effusions typical of wet FIP.

If a strong cell-mediated immune response develops in the early stage of the infection, virus replication could be lowered and infection can be limited or successfully cleared with no evidence of clinical signs. Conversely, if the cell-mediated response is weak and unable to reduce virus replication an intermediate response can also occur with a continuous release of virokines and chemokines that recruit neutrophils and macrophages into infection sites. This leads to the formation of pyogranulomas, typical of dry FIP. Dry FIP may also become effusive in the terminal stage of the disease when the immune system collapses. Thus, the development of FIP depends on the balance between humoral and cellular immune response (Pedersen, 2009). The cytokine interferon-γ (IFN-γ) is one of the main modulators of cell mediated immunity (Berg et al., 2005). IFN-γ shifts the immune response from T-helper 2 to T-helper 1 with a consequent enhancement of immune cytotoxic activity due to the activation of macrophages and CD8+ T cells. This type of immunity can protect the host against the development of the disease or even more against the infection (Pedersen, 2009).

All coronaviruses contain at least three main structural proteins: spike (S), membrane (M) and nucleocapsid (N). The nucleocapsid (N) protein is one of the most produced viral proteins during viral infections and may exert several functions. It is primarily involved in the encapsidation and packaging of viral genomic RNA; in addition to this structural role, it also participates in viral RNA transcription, replication and translation (Lai and Canavagh, 2007), and in modulating the metabolism of host cells (Eleouet et al., 2000; Surijt et al., 2006).

Several studies have identified antigenic determinants in N protein of human and animal coronaviruses and have demonstrated that N protein is one of the immunodominant antigens in the CoV family (Collisson et al., 2000; Liu et al., 2001; Zhao et al., 2007).

The N protein may play an important role in the pathogenesis of FIP. Different recombinant vaccines based on N protein have already been tested and in some cases they proved to be effective in preventing the progression of FIP. This suggests that a cell mediated immunity against the FIPV antigen was induced by the vaccine (Wasmoen et al., 1995; Hohdatsu et al., 2003) and that therefore N protein could be a suitable vaccine candidate.

The precise identification of T-cell-stimulating epitopes represents a difficult task but it is an important prerequisite for accurate epitope mapping and for formulation of vaccines and immunotherapies. A systematic approach based upon the synthesis and testing of large sets of overlapping peptides is very expensive. In contrast, bioinformatic prediction is extremely cheap and can be useful for selecting potentially immunogenic peptide regions, reducing the number of peptides to be screened. The strategy that combines the predictive power of these theoretical approaches with experimental *in vitro* or *in vivo* approaches allows a reliable and rapid identification of T cell stimulating epitopes (Schirle et al., 2001). Many web-based databases and prediction algorithms are now available and have been applied successfully to predict T-cell stimulating epitopes in infectious disease, cancer, autoimmunity and allergy.

In this study, the possible cell mediated immune response induced by N protein was investigated using both a computational prediction method and an *in vitro* assay to assess the T-helper 1 responses. Specifically, 8 potential immunogenic peptides from N protein were designed through a bioinformatic approach and the production of IFN-γ was assessed after incubation of feline leucocytes with these peptides.

2. Materials and methods

2.1. Animals and samples

The method employed to assess IFN-γ production was based on incubation of whole blood with different protein N peptides. The cell viability provided by this method was already evaluated in a previous study where it appeared greater than 95% after 1-h of incubation (Gelain et al., 2006).

Blood samples (0.8–3 ml) were collected from the jugular vein of 20 privately owned cats, after informed consent was obtained by the owners, and put into EDTA-coated tubes.

Specifically, the blood from 16 of the 20 cats (cats #1 to #16) was used to assess IFN-γ production by an ELISA system (see the details below). At the time of sampling, these cats were diagnosed as clinically healthy ($N = 7$) or showing symptoms of diseases other than FIP ($N = 9$), such as hepatic lipidosis ($N = 2$), neoplasia ($N = 2$), chronic gastroenteritis ($N = 2$), cardiopathy ($N = 1$), bone fractures ($N = 1$) and trauma ($N = 1$).

The blood from additional 4 cats (cats A–D) was used to assess the expression of mRNA coding for feline IFN-γ (see the details below). When sampled, all these 4 cats were diagnosed as clinically healthy.

For each sample a complete blood cell count (CBC) was obtained using an automated haematology analyser (Sysmex XT-2000iV) together with a manual differential leukocyte count performed on May-Grunwald Giemsa stained smears. An aliquot of each sample (200 μl) was then centrifuged (8 min at $450 \times g$) and plasma was harvested and transferred to Eppendorf plain tubes for FCoV serology and, in 10 cases, for the assessment of the baseline (pre-incubation) value of IFN-γ. Specifically, the presence of anti-FCoV antibody titres was investigated using an indirect immunofluorescence test performed on multiwell slides produced at the University of Zurich according to Osterhaus et al. (1977) and using the procedure routinely performed in our laboratory (Paltrinieri et al., 2008). The concentration of IFN-γ was measured with the method described below.

To assess the possible non specific influence of peptides on IFN-γ measurement by the ELISA technique described below, a pool of fresh feline plasma from blood samples routinely submitted to our Institution was also included in the study in addition to the blood samples described above, as negative control.