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Chapter 1

THE ROLE OF CORTISOL IN FELINE RETROVIROSIS

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ABSTRACT

In this chapter we review the role of cortisol in the pathogenesis of chronic viral infections, such as feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV). Cortisol is the main hormone involved in the "fight or flight" response, as it is released in response to stress. Its primary functions are to increase blood sugar through gluconeogenesis, aid the metabolism of fat, protein and carbohydrate, and decreases bone formation. Its effects on the immune system are also well known. It inhibits the production of interleukin (IL)-12, interferon (IFN)-γ, IFN-α and tumor-necrosis-factor (TNF)-α by antigen presenting cells and T helper (Th)1 cells, but upregulates IL-4, IL-10 and IL-13 by Th2 cells.

This results in a shift towards a Th2 immune response rather that general immunosuppression.

All these effects have great importance in the pathogenesis of chronic viral infections, such as by herpes viruses or retroviral infections. Feline retroviruses are important pathogens of cats. Both feline immunodeficiency virus (FIV) and feline leukemia virus (FeLV) induce life-long lasting diseases in cats, which are characterized by the establishment of non-specific clinical signs (such as gingivitis, weight loss, cachexia, etc.) which might be a consequence of high cortisol levels. In fact, we have found that both FeLV-positive and FIV-positive cats have significantly increased cortisol plasma levels when compared to non-infected cats, but significantly lower than animals infected by both viruses. These high cortisol levels determine the shift of the immune response from Th1-type (adequate for fighting intracellular microorganisms) to Th2-type (associated with a poor prognosis).

As other steroids, cortisol is able to diffuse through the cell membrane into the cytoplasm, where it binds to glucocorticoid receptors (GR). Homodimer GR-cortisol complexes are translocated to the nucleus, where they bind specific DNA responsive elements (GRE) activating gene transcription. A second mechanism involves the binding of GR-cortisol to NF-κB or AP-1 to repress them from transactivating target genes. These two mechanisms are called transactivation and transrepression, respectively. They are responsible for the biological effects of cortisol, which depend also on the type of cell exposed to the steroid. For example, when GR-cortisol complexes cross the nuclear membrane of Th cells, they bind GRE which transactivate the IL-4, IL-10 or IL-13 genes and differentiate the lymphocyte into a Th2 cell.

Interestingly, retroviruses possess DNA sequences which act as GRE, i.e., they bind GR-cortisol complexes. They are located in the long terminal repeats (LTR), which are sequences which flank the proviral DNA integrated in cellular genome. LTR control viral transcription precisely by binding transcription factors, such as GR-cortisol. In other words, the virus is expressed in response to the cortisol levels in the cellular environment. Reduction in cortisolemia could be beneficial in these retroviral infections, but more studies are still needed to understand better the effects of cortisol in feline retrovirosis.

INTRODUCTION

Cortisol plays a relevant role in several infectious diseases. Cortisol is a steroid hormone, more specifically a glucocorticoid, produced by the cortex (from where the name is derived) of the adrenal gland in the zona fasciculate.

As the other steroid hormones, it derives from cholesterol. Cortisol is the main hormone involved in the "fight or flight" response, as it is released in response to stress. Its primary functions are to increase blood sugar through gluconeogenesis and glycogenolysis. It participates in the metabolism of lipids, proteins and carbohydrates. It also decreases bone formation, and suppresses the immune system. These functions are also shared by other glucocorticoids (GCs) such as cortisone or corticosterone and the synthetic GCs hydrocortisone and dexamethasone.

The synthesis of steroid hormones in the glands is controlled by the neuroendocrine axis formed by the hypothalamus and the pituitary gland, which secrete adreno-cortico-tropic hormone (ACTH) to regulate the hypothalamus-pituitary-adrenal gland (HPA) axis, or the hypothalamus-pituitary-gonadal (HPG) axis (Tejerizo et al., 2012). In the case of cortisol, the hypothalamus secretes corticotrophin-releasing hormone (CRH), which triggers cells in the neighboring anterior pituitary to secrete another hormone, ACTH. ACTH is carried through blood to the adrenal cortex where it increases the concentration of cholesterol in the inner mitochondrial membrane and stimulates the synthesis of cortisol and other glucocorticoids, mineralcorticoids and dehydroepiandrosterone (DHEA) (Figure 1). The HPA axis, as well as the HPG axis, is susceptible of being altered by different signals, either exogenous to the body or stemming from other systems, such as neurotransmitters or cytokines. In this way, the neurological, endocrine and immune systems are tightly interconnected (Figure 2). Each of the elements possesses its own molecular network of internal regulation, and the interaction with these signals may lead to its deregulation and the alteration of its functions (Gomez-Lucia et al., 2007).

Cortisol plays an important role in providing the body with a readily available energy source, glucose, from stored reserves by two different mechanisms: gluconeogenesis and glucogenolysis. In the fasting state, cortisol stimulates gluconeogenesis (formation of glucose, in the liver, from certain amino acids, glycerol, lactate, and/or propionate), inhibiting the synthesis of proteins. In other circumstances, cortisol promotes glycogenolysis, the breaking down of glycogen to glucose-1-phosphate and glucose, in liver and muscle tissue. Both mechanisms increase the amount of blood glucose available for fuel by the muscle, and allow the body to react to immediate danger or stress. However, prolonged cortisol secretion (which may be due to chronic stress) results in significant physiological changes.

Elevated levels of cortisol, if prolonged, can lead to proteolysis and muscle wasting. Its effects on lipid metabolism are controversial.

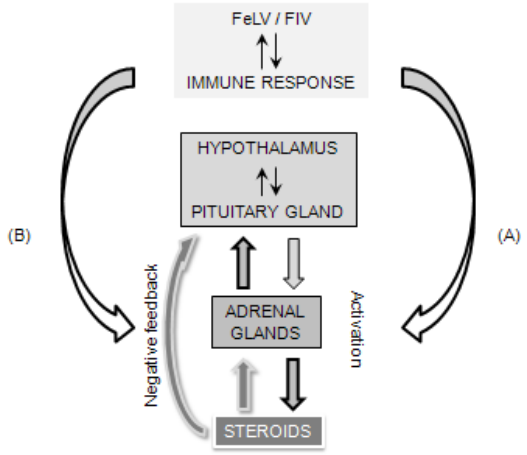

Figure 1. Possible interferences of FeLV and FIV with the HPA route. The relationship between the virus and the immune response of the cat could affect the activation routes (A), or the negative feedback circuits (B). Both events would affect the final concentration of hormones.

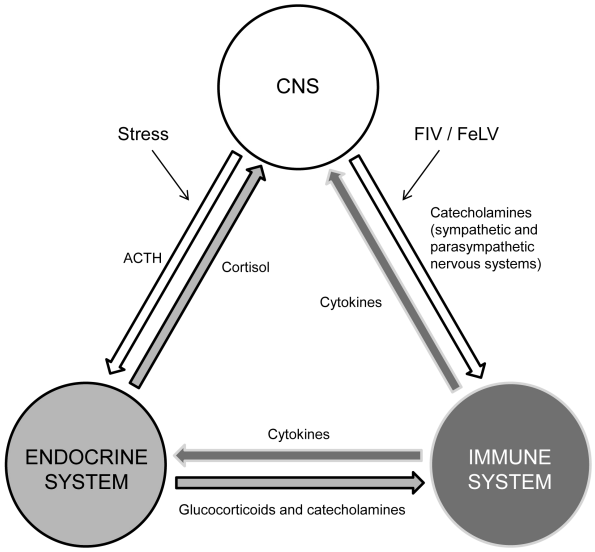

Figure 2. Network of three-directional communication between the immune, nervous and endocrine systems. Stress or an infection can activate and modify the proinflammatory cytokines balance, and stimulate bothe the hypothalamus-pituitary-adrenal axis (HPA), as well as the sympathetic-adrenal system. This induces the release of ACTH, glucocorticoids and catecholamines (adrenaline and noradrenaline) that affect the immune response.

Several studies have shown a lipolytic effect of cortisol; however, under some conditions, cortisol may somewhat suppress lipolysis (Bhathena, 2006; Mattsson and Olsson, 2007).

The effects of cortisol on the immune system are also well known. An important characteristic of cortisol and other GCs is their potent anti-inflammatory ability, repressing the migration of leukocytes and the activity of lymphocytes. GCs also reduce the secretion of histamine and stabilize lysosomal membranes, preventing their rupture and avoiding damage to healthy tissues (Yoffe, 1981).

Cortisol inhibits the production of interleukin (IL)-12, interferon (IFN)-γ, IFN-α and tumor-necrosis-factor (TNF) α by antigen presenting cells and T helper (Th)1 cells. On the other hand, it upregulates IL-4, IL-10 and IL-13 secretion by Th2 cells. This results in a shift towards a Th2 immune response rather that general immunosuppression. In addition, it prevents the proliferation of T-cells by rendering the IL-2 producer T-cells unresponsive to IL-1 and unable to produce the T-cell growth factor (TCGF) (Dimitrov et al., 2004). The activation of the stress system (and resulting increase in cortisol and Th2 shift) seen during an infection is believed to be a protective mechanism which prevents an over activation of the inflammatory response.

For all these reasons, GCs are used to treat inflammatory diseases resulting from over activity of the B-cell-mediated antibody response. Examples include inflammatory and rheumatoid diseases, as well as allergies, but also certain types of leukemia and lymphomas (Gomez-Lucia et al., 2007).

FELINE LEUKEMIA AND FELINE IMMUNODEFICIENCY

Both feline immunodeficiency and feline leukemia have aspects in common: they are chronic and persistent infections affecting mainly lymphocytes and other cells of the immune system, and are characterized by a variety of signs that are clinically nonspecific. Besides, the excess of circulating immune complexes which develop in both infections could contribute to the immune mediated lesions and signs associated with the infection by any of these two viruses. The clinical signs are further confused by the possibility of animals infected by both viruses or double infected. In spite of these similarities, the pathogenic mechanisms of both viruses are quite different and not completely understood yet.

FIV usually is transmitted through bites and wounds produced by aggressions between cats. The infection progresses slowly through several

stages during several months or even years, although there is no clear distinction between these stages in naturally infected cats, and in most animals it does not adversely affect the cats' life expectancy (Hartmann, 2012). After an initial replication in the regional lymph nodes and other lymphoid organs, a short initial viremia occurs in which the virus is spread throughout the body, infecting lymphocytes, monocytes and macrophages of the bone marrow, intestinal tract, lung, kidney and brain ("initial stage" or "acute phase"). A few weeks later, the cat develops a cellular and humoral immune response which, however, fails to completely eliminate the virus. This acute phase, which generally lasts four to six weeks but can persist up to one year, is characterized by transient or mild clinical signs, as hyperthermia, lethargy, generalized lymphadenopathy, stomatitis, diarrhea, depression, skin lesions, intraocular inflammation, variable neurologic signs, etc. (Domenech et al., 2012; Lawson and Hosie, 2001).

The infection progresses slowly to a chronic subclinical period, which corresponds to the "phase of clinical latency", with low levels of viral expression. This period can last from months to many years and even may be lifelong lasting in some animals. During this subclinical phase, the immune system weakens gradually, with a progressive decrease of $CD4^+$ T-cells (due to direct replication of the virus in these cells) which leads to lymphopenia and inversion of the CD4/CD8 ratio, along with a decrease in the functionality of infected lymphocytes. In most FIV-infected cats, there is a marked hypergammaglobulinemia, primarily due to the polyclonal stimulation of B-cells which give rise to an increase of IgG not specific to FIV (Miró et al., 2007). As infection progresses, mild clinical signs, such as relapsing fever, leucopenia, lymphadenopathy, anorexia, weight loss, etc. may develop. At the end of this stage, the clinical triad lymphadenopathy-gingivitis-hypergammaglobulinemia may be observed (Collado et al., 2012; Domenech et al., 2012; Gleich and Hartmann, 2009; Hartmann, 2011, 2012).

In some infected cats, often many years after infection and coinciding with very low values of CD4/CD8, plasma viremia increases, leading to a terminal phase, the so-called "feline acquired immunodeficiency syndrome" or FAIDS, due to its resemblance to human AIDS (Hartmann, 2011, 2012). During this stage with clinical signs the lymphocyte depletion is much more pronounced and opportunistic infections occur, against which the cat cannot develop an adequate immune response. Multiple chronic infections are observed, such as gingivo-stomatitis, respiratory diseases, pyodermas, or disorders of the nervous system, the kidneys, the eyes, etc. When the CD4/CD8 ratio decreases further, acquired immunodeficiency develops, and tumors, such as squamous

cells carcinoma and lymphoma, neurologic and several immune-mediated clinical signs may be detected (uveitis, glomerulonephritis, cholangitis, etc.), although very few are due to direct viral action (Hartmann, 2011, 2012; Lawson and Hosie, 2001). Cats which suffer FAIDS die from a chronic syndrome of wasting, neurological disease, neoplasia or systemic opportunistic infections (Domenech et al., 2012; Dunham and Graham, 2008; Hartmann, 2011, 2012).

Saliva is the main source of infection for FeLV, as viremic cats with or without clinical signs shed constantly viral particles through it (Hartmann, 2011). For this reason, mutual grooming or sharing food or water bowls are risk factors for FeLV transmission. The animal usually gets infected by the oronasal route, and the virus replicates in the local oropharyngeal lymphoid tissue. The outcome of FeLV infection is very different in each cat, as it depends on factors related to both the virus (subgroup of FeLV, duration of exposure and infective viral load) and the host (age and immune status) (Dunham and Graham, 2008; Hartmann, 2011). Most cats develop an effective immune response, but in very few of them the virus is completely cleared from the body at a very early stage ("abortive infection") and animals never become viremic. After the initial replication, there is a transient viremia in which the virus infects lymphocytes and monocytes, and is spread to different tissues (spleen, thymus, salivary glands, lymph nodes) ("regressive infection"). However, in some cats viremia persists longer than three weeks and FeLV infects bone marrow stem cells, producing a massive replication of the virus and the development of persistent viremia (both of free virus and of infected cells), which gives rise to "persistent or progressive infection". In this phase, the virus infection cannot be completely eliminated because the provirus is present in the stem cells in the bone marrow. In addition, hematopoietic alterations (particularly cytopenias) are produced, caused by the suppression of the bone marrow as a result of the infection (Hartmann, 2011, 2012). An unknown percentage of cats develop a "latent infection", in which, although the bone marrow becomes infected, it does not release virus or infected cells. These animals remain apparently healthy until the provirus is reactivated by an immunosuppressor or treatment. Very young cats are more likely to develop a progressive infection, probably due to their immature immune system, while older cats tend to develop a regressive or abortive infection. Atypical infections in which the virus is located in organs, as the bladder, eyes or the mammary gland, partially hidden from an effective immune response, have been described in a very low number of animals.

The clinical signs in FeLV persistently infected cats are more specific than those observed in the course of FIV infection and the evolution is faster, and these cats usually die in 1-3 years from a disease related to FeLV infection, including neoplastic and/or non-neoplastic disease. FeLV is an oncogenic virus which may induce several types of neoplasias, of which lymphoma and lymphoblastic leukemia are the most prevalent. Besides that, myeloproliferative neoplasias, fibrosarcoma, osteochondroma and neuroblastoma have also been described in infected cats (Collado et al., 2012; Hartmann, 2011, 2012; Hofmann-Lehmann et al., 2007).

Non-neoplastic diseases include hematologic disorders, immunesuppression, immune-mediated diseases and other syndromes, as neuropathy and reproductive disorders. Anemia is frequently observed in viremic cats and could be predictive of imminent death. It may be non-regenerative (as a result of myelosuppression, myelodestruction, or myeloproliferative diseases) or hemolytic (immune-mediated or secondary to *Mycoplasma haemofelis* infection) (Hartmann, 2011; Hofmann-Lehmann et al., 2007). Lymphonenia and neutropenia are also common (Collado et al., 2012). Immunosuppression may be caused by different mechanisms and it is mainly associated with some variants of the virus. Certain immunosuppressive FeLV strains (FeLV-T), with a strong tropism for T-cells, have also been reported to induce an acquired immunodeficiency syndrome similar to the one produced by FIV (Hartmann, 2011). The immunosuppression predisposes animals to secondary infections, such as stomatitis, abscesses, pyothorax, dermatitis, feline infectious peritonitis, toxoplasmosis or cryptococcosis. Some immune-mediated alterations observed in FeLV positive cats are autoimmune glomerulonefritis, uveitis and neutrophilic polyarthritis (Hartmann, 2011; Hofmann-Lehmann et al., 2007). In regards to reproductive disorders, FeLV infection may also cause infertility and abortions, and newborn kittens are frequently lethargic. Nowadays, most of persistently infected cats show anemia, immunosuppresive or immune-mediated disorders instead of tumors (Hartmann, 2011).

Both retroviral infections in cats induce deep deregulation of the functions of the immune system, probably associated with changes in the cytokine pattern, especially described in FIV infections. Increased TNFα and altered levels of IFN-γ, IL-2, IL-4, IL-6 are observed in FeLV and FIV infected cats (Hartmann, 2011; Levy et al., 2004; Linenberger and Deng, 1999). Proinflammatory cytokines (IL-6, TNFα) may also contribute to illness. Increased IL-10/IL-12 ratio (due to an increase of IL-10) found in FIV infection is associated to the shift from type Th1 to response (which restrains disease progression) to type Th2 (which allows disease progression) during

FIV progression (Levy et al., 2004, 2004). The excessive production of the Th2 type cytokine IL-10 may also induce the production of glucocorticoids indirectly (via the release of cortisol releasing hormone, CRH, and/or ACTH) or directly by stimulating adrenal glucocorticoid biosynthesis, which could be also associated with the evolution of infection. As cortisol suppresses cell-mediated immunity and may stimulate humoral immunity, its increase could be detrimental in FIV infection.

CORTISOL IN FIV AND FELV INFECTIONS

In general, viral infections increase cortisol levels through activation of the HPA axis by cytokines (Silverman et al., 2005). Infections by FIV or FeLV are no exception to this. Diseases produced by these pathogens are also paradigmatic in the sense that they are chronic, long-lasting infections, which produce a continuous activation of the immune system. However, there are few studies which focus on hormonal alterations resulting from infection by feline retroviruses. Teng (1990) reported both increases and decreases in the plasma concentration of cortisol and ACTH in FeLV-infected cats, which depended on the stage of the disease. They were attributed to the absence of adrenal response to the pituitary orders.

In the case of FIV, to the best of our knowledge, these types of studies are non-existent. Recently, our research group has obtained the results shown below.

Cortisol levels were determined in the serum or plasma of cats naturally infected by FIV (9 female and 33 male cats), FeLV (26 female and 17 male cats), or FIV and FeLV-infected (6 female and 6 male cats), as well as non-infected (38 females and 35 male cats) using a competitive EIA technique (Tejerizo et al., 2012). Retroviral infection was diagnosed using Snap Combo (Idexx) and a double-nested PCR designed by our group (Arjona et al., 2007). Statistically significant differences in cortisol levels between infected and non-infected cats were observed. Cortisol levels in non-infected, FeLV-positive (FeLV+), FIV-positive (FIV+) and FeLV and FIV-positive (F-F) cats were an average of 53, 213, 217 and 662 ng/mL, respectively; *i.e.* 4x higher in cats infected by either FIV or FeLV and 12x in double-infected cats than in non-infected animals (Figure 3A). Generally, infected male cats had higher cortisol plasma concentrations than infected female cats (Figure 3B) (differences between males and females were statistically significant, $p<0.05$, in FeLV+ cats). FIV-infected cats with clinical signs had lower cortisol plasma

concentration than FIV-infected cats without clinical signs, but differences were not statistically significant (Figure 3C). The highest cortisol concentrations (850-1290 ng/mL) were found in three FIV+ cats, but no pattern according to sex, age, or presence/absence of clinical signs could be established. The small range of variation of the concentration of plasma cortisol in each of the groups (non-infected, FeLV+, FIV+, F-F; Table 1) was surprising. This was regardless of the time of the day in which the blood samples were taken, the vaccination status, stress, being stray or home-kept, or any other circumstance which usually affects cortisol concentration.

Diurnal cycles of cortisol levels are found in several animal species. In species that exhibit such cycles, different timing of diurnal maxima and minima has been observed, not only in different species (Martin, P.A. and Crump, M.H., 2003) but also, in some cases, within the same species (Fulkerson and Tang, 1979; Mesbah and Brudieux, 1982; Simonetta et al., 1991; Stella et al., 2013). The variations are due to the information about the light/dark cycles, transmitted from the retina.

Though there are few data about cortisol levels in FIV-infected cats, cortisol concentrations have been analyzed in individuals infected with the closely related lentivirus HIV. Endocrine dysfunctions have also been observed in HIV-positive patients. It is known that in the case of HIV many of the clinical signs associated to the infection are similar to those derived from an excessive production of GCs. In general, with a few exceptions, data show a clear increase of cortisol and decrease of dehydro-epi-androsterone (DHEA) concentrations in all HIV-1 patients, regardless of the stage of the disease and the treatment administered (Abrams et al., 2007; Bons et al., 2013; Chittiprol et al., 2007, 2009; Christeff et al., 2000, 2002a; Clerici et al., 2000; Laudat et al., 1995; Schifitto et al., 2000). This phenomenon seems to be accompanied by high levels of estrogens (Christeff et al., 1992; Poretsky et al., 2009; Teichmann et al., 1998) and reduced levels of testosterone (Christeff et al., 1992; Laudat et al., 1995; Poretsky et al., 1995; Villette et al., 1990).

CORTISOL / DHEA RATIO

DHEA is another steroid hormone, secreted by the adrenal cortex, the gonads and the brain, where it functions predominantly as a metabolic intermediate in the biosynthesis of androgen and estrogen sex steroids (Domenech et al., 2009).

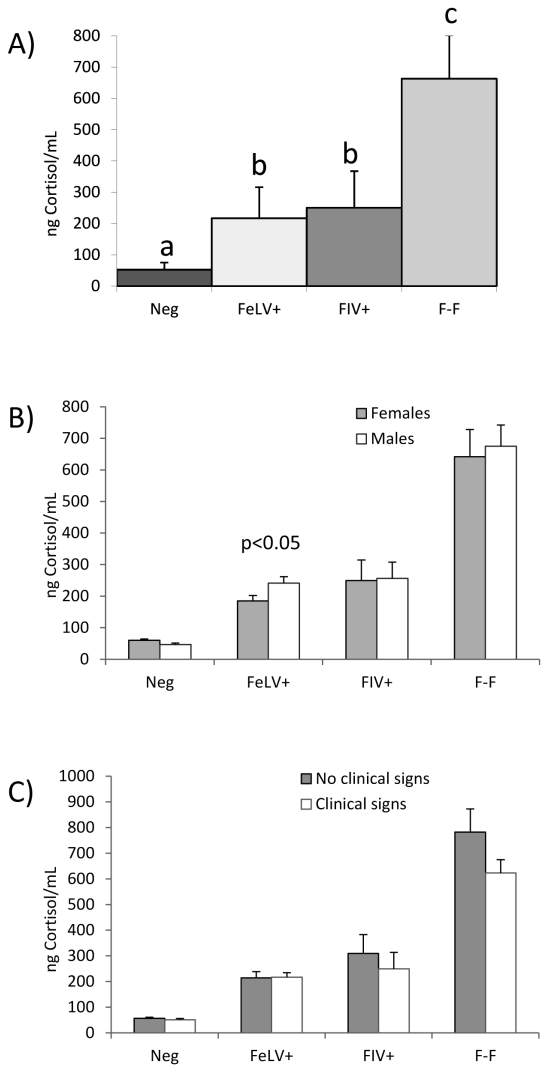

Figure 3. Concentration of plasma cortisol in retrovirus-infected and non-infected cats. A) Concentration of cortisol in each study group; groups identified with the same letter do not have statistically significant differences between them, while groups with different letters have statistically significant differences ($p<0.05$) between them. B) Concentration of cortisol in each study group in relation to the sex of the cats. C) Concentration of cortisol in each study group in relation to the presence or absence of clinical signs in cats. Neg, non-infected cats; FeLV+, cats infected by FeLV; FIV+, cats infected by FIV; F-F, double infected cats.

Table 1. Concentration of cortisol and DHEA expressed in ng/mL in the four groups of cats considered. SD, standard deviation

	Cortisol (SD)	**DHEA (SD)**	**Cortisol/DHEA**
Negative	53 (4.24)	10.4 (0.46)	5.1
FeLV-positive	213 (17.5)	5.9 (0.62)	36.3
FIV-positive	217 (58.4)	5.0 (0.34)	43.1
FeLV and FIV-positive	662 (76.7)	1.8 (0.62)	358.8

DHEA is the main and most abundant adrenal androgen. It has been associated with multiple beneficial effects for the body, though its mechanism of action and whether the hormone itself or its subproducts are responsible for these effects is still greatly unknown. Conceivably, the effects would be exerted through binding to an array of nuclear and cell surface receptors, and acting as a neurosteroid. DHEA is thought to interfere with the biosynthesis of cortisol through the enzyme 11β-HSD1, which has been suggested to be involved in the anti-glucocorticoid effects of DHEA (Muller et al., 2006), antagonizing by this route the immune suppressive effects of GCs.

DHEA has been shown to inhibit the replication of HIV-1 *in vitro* (Henderson et al., 1992) and to inhibit the reactivation of the latency of HIV-1 (Yang et al., 1993). Low levels of this hormone would be associated with a higher risk of progression to the most severe form of AIDS. The inhibitory effect of DHEA on FIV replication has also been shown (Bradley et al., 1995; Pedersen et al., 2003). In fact, DHEA has been administered to FIV-infected cats (Pedersen et al., 2003) and to patients with AIDS (Abrams et al., 2007; Rabkin et al., 2006) to boost their cell mediated immunity. In women, DHEA levels are higher than in men, and it could be a mechanism to explain the higher progression rate to AIDS in men than in women. In general, the reduction in $CD4^+$ cell counts is associated with the decrease in the levels of both DHEA and its derivate, DHEAS in both infected men and women (Chittiprol et al., 2009; Christeff et al., 2000; Laudat et al., 1995; Maingat et al., 2012; Schifitto et al., 2000; Zapanti et al., 2008). The increase in the cortisol/DHEA ratio could contribute, at least in part, to two fundamental facts in the pathogenesis of the HIV infection: the altered cytokine pattern and immune function, and the critical loss of lean body mass in patients with wasting syndrome. Lipodistrophy correlates with serum DHEA and increased cortisol/DHEA ratio, and with IFN-α levels in HIV-1-infected patients (Andersen et al., 2007; Christeff et al., 2000, 2002b; Norbiato et al., 2000; Zapanti et al., 2008).

The same cats as mentioned above were analyzed for the plasma or serum concentration of DHEA (Tejerizo et al., 2012). The values obtained are shown in Table 1. A similar trend to what is observed in HIV-1 infection could be seen in cats infected with FeLV or FIV. The concentration of plasma DHEA was significantly higher in the negative cats than in the infected cats, and in cats with monoinfection (either FIV or FeLV) than with dual infection. However, in our study the plasma concentration of DHEA in retrovirus-infected cats did not correlate with the presence or absence of clinical disease, unlike previously reported data on HIV infection (Christeff et al., 2000), but FIV-infected females had higher plasma levels of DHEA than males. This higher plasma levels of DHEA could be somehow involved in a greater survival of queens with respect to male cats that we detected earlier (Arjona et al., 2000). In general, it seems that the infection and disease stages are extremely important in the hormonal deregulation associated with retroviruses. Unfortunately, due to the highly dispersed origin of our samples, we could not do a follow-up of the animals, characterize in detail their clinical status, or analyze the stage of the infection at the time of blood collection. The wide range of possibilities associated with such non-specific clinical signs which characterize these infections, only allowed us to classify cats with and without clinical signs.

One of the most obvious consequences of the increase in plasma cortisol levels is the effect on the cytokine patterns. A shift from type-1 to type-2 cytokine production is detected in most HIV-1 patients during disease progression. This shift includes a defective production of IFN-γ, IL-2, and IL-12 along with the increased production of IL-4, IL-5, IL-6 and IL-10. GCs and IL-4 stimulate the differentiation of B lymphocytes into IgE-producing plasma cells, the concentration of which augments in HIV infection (Clerici et al., 2000).

MECHANISM OF CORTISOL DEREGULATION

The mechanisms by which viral infection causes this hormonal deregulation are still not clear and have been analyzed mostly in HIV infection. Although the mechanisms leading to adverse effects on HPA axis activity in HIV infection are not fully understood, several lines of evidence suggest that a number of mechanisms may be involved, including homologies in molecular structures of various mediators of neuroendocrine activity and

HIV-related structures, HIV as a chronic stress model, and virus-induced toxic factors (Kumar et al., 2002).

A possibility could be that the immune response, activated to fight the virus, triggers several signaling pathways, mainly mediated by cytokines, which could interfere with the regulatory mechanisms of the neuroendocrine system (*indirect regulation*). On the other hand, the infection could be interfering with the endocrine routes in a direct way, through the interaction of the virus or its products with elements of the neuroendocrine system (for example, infection in the hypothalamus) (*direct regulation*). In both cases, the virus could also interact with the end components of the HPA axis, the adrenal glands, without affecting the other elements of the endocrine control. Data supporting either route are discussed below.

In the case of *indirect regulation*, mediated by the activation of the immune response, it has been shown that both FeLV and FIV infections lead to an increase in the expression of proinflammatory cytokines such as IL-1, IL-6, TNFα (Gómez et al., 2011; Lawrence et al., 1995; Peterson and Chesebro, 2006; Robert-Tissot et al., 2011), and can activate the HPA axis (Besedovsky and del Rey, 1996). This activation would lead to an increase in levels of circulating GCs as has been shown for HIV-1 (Clerici et al., 2000; George and Bhangoo, 2013). Alterations in the production of adrenal steroids and a complex pattern of deregulation in cytokine profiles accompany the progression of HIV infection (Clerici et al., 2000). It has been observed that in some viral infections (*eg*.: produced by cytomegalovirus, or Newcastle disease paramyxovirus) the presence of certain cytokines, such as IL-1 or IL-6, can be decisive for the activation of this circuit (Dunn and Vickers, 1994; Ruzek et al., 1997). Although these interactions are known in acute phase responses, it has not been determined what happens really in cases of continuous stimulation or in conditions of chronic pathologies, as could be the case of retroviral infections. Numerous observations endorse also the hypothesis of a *direct regulation* of the HPA axis by the virus or its proteins. These viruses and other related viruses, such as HIV, are known to cross the brain-blood barrier and invade the central nervous system (CNS) and cause neurological damages in the host (Carmichael et al., 2002; Fletcher et al., 2008, 2009; Koirala et al., 2000; Podell et al., 2000; Ryan et al., 2005; Vahlenkamp et al., 1999). The presence of the virus within the control centers could alter the activation routes of the hypothalamus-pituitary axis and modify the endocrine regulation of the HPA axis, leading to the production of anomalous hormone levels. In this sense, the presence of FeLV or its proteins in the hypothalamus has been linked to an endocrine deregulation, affecting the concentrations of

growth hormone (Tshikuka et al., 1992) or the HPA circuit in general (Wang and Teng, 1994). Cytoplasmic viral antigens have been detected by indirect immunofluorescence assays in the fibers of hypothalamic preoptic region of FeLV-infected cats (Wang and Teng, 1994). A similar dysfunction could be due to the presence of FIV in these regulating elements. The neurological damages associated to the lentiviral infection are partly mediated by the neurotoxicity of the envelope protein of the virus (Johnston et al., 2002; Power et al., 2004). Data supporting this are: a) It has been shown that the expression of HIV gp120 in the brain, frequently detected in AIDS patients, increases the concentrations of CRH, ACTH and cortisol (Costa et al., 2000; Raber et al., 1996); b) Treatment with GCs worsens the neurological damage produced by this viral protein (Brooke and Sapolsky, 2000, 2002); c) In connection with the indirect effects mentioned in the previous paragraph, encephalitis associated to the infection by HIV leads to an increase in the levels of proinflammatory cytokines, such as IL-1, IL-6, and TNFα (Persidsky et al., 1997). Altogether, these data could endorse the capacity of FeLV or FIV of interacting directly with neuroendocrine mechanisms of regulation.

Another possibility is the direct interaction between the *adrenal gland* and the retrovirus or cytokines secreted as a result of the infection, independently of initial elements of the regulating axis. This would induce a direct increase in the levels of GCs without the involvement of the hypothalamus or the pituitary gland. This has been demonstrated in other viral infections, such as infections by murine cytomegalovirus (Ruzek et al., 1997) or by Newcastle disease virus (Smith et al., 1982), and in certain cases of sepsis and chronic and autoimmune diseases. Mechanisms involving other elements are also possible. For example, in some HIV-positive patients who develop AIDS, the ability of cortisol to bind the cortisol binding globulin (CBG), responsible for the transportation of the hormone to its target tissues, is affected. This could limit even more the functional ability of the GCs (Martin et al., 1992).

The result of this inability of CBG is a continued activation of HPA axis and an increasing concentration of GCs unable to control their own production by feedback mechanisms.

CONSEQUENCES OF THE CORTISOL UNBALANCE

The neuroendocrine axis is indisputably essential to recover the basal homeostasis of the host after the infection (Ruzek et al., 1997; Silverman et al., 2005). Both FeLV and FIV could have acquired certain evolutionary

advantage when interacting with this system, because they could benefit from the immunosuppression associated to its chronic activation. In addition, FIV and FeLV would also benefit from the shift of the Th1- to the Th2-type response mediated by cytokines in response to GCs. This shift involves an excess of antibodies and a weak cellular response. An effective defense against these intracellular pathogens needs to be mediated by a strong cellular immunity.

In our study, the cortisol levels were significantly higher and DHEA levels were significantly lower in double-infected cats than in cats with monoviral infection. Two hypotheses could explain this observation. If some infected animals were especially immune depressed, due to a greater alteration in the cortisol/DHEA ratio, they could be more susceptible to other pathogens, including the other retrovirus (FIV in the case of initial FeLV infection and vice versa). A second possibility could be that the infection by a second retrovirus would affect more markedly the concentration of both hormones. As we discussed previously, in the cortisol/DHEA balance it is difficult to differentiate the cause from the effect and to determine whether the unbalance is due to the retroviral infection or it derives from the physiological system of stress against infection. It has been proposed that GR could be a key protein exploited by HIV at multiple levels to ensure its pathogenic success (Hapgood and Tomasicchio, 2010). Besides the evident direct effect on the HPA axis of the viral infection and the immune system triggered by it, other conditions, concomitant to the infection, such as pain, weakening, or physiological stress may also participate actively in the activation of the HPA axis, increasing the GCs levels and making it even more difficult to establish the role of each component in the endocrinal regulation.

TREATMENT WITH CORTICOIDS

The interaction between retrovirus and the HPA axis has serious *clinical implications*. GCs are routinely included in therapeutic protocols for retrovirosis as depressants of the immune response to revert the complications associated to the retroviral infection, such as neuropathies, renal disease, skin exanthema, gut inflammation, reactivation of a clinical toxoplasmosis (Lin et al., 1992), Burkitt lymphoma or pneumonia by *Pneumocystis carinii* (Barr et al., 2000). Treatment with GCs, such as dexamethasone or prednisolone, could be detrimental for an already immune suppressed host, in spite of their powerful anti-inflammatory effect. In these cases a therapeutic intervention

aimed to diminish the endogenous levels of GCs would be more effective. The detrimental effect of steroid therapy has been shown in FeLV- or FIV-infected cats. Cats treated with GCs before being infected experimentally with FeLV lost their natural resistance to persistent viremia (Rojko et al., 1988). In the case of FIV, it has been shown that the administration of GCs increases the levels of viral ARN in plasma (Barr et al., 2000). Therefore, the effects of GC administration should be a factor to consider when choosing the most suitable therapy for retroviral infection. In addition, it has been reported that antiretroviral therapy may increase cortisol levels in HIV-1 infected patients (Collazos et al., 2004). However, this does not seem to be the case in FIV-infected treated cats (at least with zidovudine) (Gómez et al., 2011).

In conclusion, the possible alterations of the levels of cortisol during the infection by feline retroviruses have not received so far the attention they deserve. According to our data, concentration of cortisol is greatly increased in FIV, FeLV or FIV and FeLV-infected animals. The fact that in double-infected animals levels are three times higher than in the single infection suggests a different mechanism of action of cortisol for both viruses. It is difficult to know whether the host activates mechanisms to increase the levels of cortisol to fight the retroviral infection, or FIV and FeLV induce this increase. Undoubtedly, elevated cortisol levels have an impact on the feline homeostasis, as it is an immunosuppressor, acts on innate immunity, lymphocyte proliferation and cytotoxicity and antigen presentation. Most importantly, it alters the cytokine expression promoting the shift of Th1 to Th2 immune responses. All these are effects of cortisol at the organic level; the next question to address is what the implications at the cellular level are.

EFFECT OF CORTISOL AT THE CELLULAR LEVEL

One of the parameters in which cortisol could be affecting at the cellular level would be apoptosis. Steroid hormones have been reported previously to induce apoptosis of lymphocytes of FeLV-infected cats (Rojko et al., 1979), FIV-infected cats (Hofmann-Lehmann et al., 1998), or FeLV-infected cells in culture (Tejerizo et al., 2005). To determine whether cortisol would also produce a similar effect, FeLV- or FIV-infected cells were exposed to serial dilutions of cortisol or dexamethasone (a potent synthetic glucocorticoid) (10^{-3}M to 10^{-12}M). After 24 h or 48 h, cells were stained with Annexin V-Fluos (Annex) and propidium iodide (PI) to differentiate apoptotic cells (Annex +, PI-) from dead cells (Annex+ or -, PI+) and evaluated by flow

cytometry. Cell-free supernatants were used to determine RT Activity and FeLV-p27 or FIV-p24 as described by Tejerizo et al. (2005).

In general, both cortisol and dexamethasone induced apoptosis of FeLV-infected cells at high concentrations (mostly 10^{-3}M) after 24 h and 48 h exposure, but the effect was noticeable in a wide range of concentrations, up till at least 10^{-7}M (Figure 5A, 5C, 5E, 5G). The effect of these GCs on FIV-infected cells was less marked. After 48 h exposure to cortisol and dexamethasone, increased apoptosis was observed in the range between 10^{-5}M to 10^{-8}M (Figure 6A, 6C, 6E, 6G). The ability of the GCs to induce apoptosis and regulate proliferation has been described previously in a multitude of cell types, including cells of the immune system (Amsterdam and Sasson, 2002; Webb et al., 2003) and in relation to tumor progression involving c-Myb (Sarvaiya et al., 2012).

In regards to feline retroviruses, although FeLV and FIV have been shown to induce apoptosis in lymphoid cells (Bull et al., 2004; Rojko and Kociba, 1991; Rojko et al., 1988, 1979, 1992; Sprague et al., 2010; Tompkins et al., 2002), the phenomenon has not been analyzed extensively in the presence of GCs. Barr et al. (2000) detected in *in vivo* studies a decline in lymphocyte subpopulations in FIV-infected cats treated with prednisolone, without significant alterations in the CD4/CD8 ratio.

The mechanisms involved in this decline were not examined and other processes related to hormone action may be involved. Other steroid hormones induce apoptosis. For example, the group of Hoffmann-Lehmann found differences in the apoptosis rate in males and females infected by FIV, and these correlated to the blood levels of 17β-estradiol and progesterone (Hoffmann-Lehmann et al., 1998). The apoptotic activity of both 17β-estradiol and progesterone has also been shown on FeLV-infected cells (Tejerizo et al., 2005). In other viruses, GCs may protect from apoptosis. Thus, it has been shown that GCs improve the survival of HIV-infected T cells by repressing apoptosis (Lu et al., 1995). However, other studies have shown that cortisol and HIV-1 gp120 act synergistically to induce apoptosis of lymphocytes from normal donors as demonstrated by DNA ladder formation and other techniques, and this was more significant in the $CD4^+$ population than in the CD47 population (Clerici et al., 2000; Nair et al., 2000). This GC-induced apoptosis of mature T lymphocytes may be responsible, at least in part, of the pathogenesis of HIV-1. Because (1) Th1 but not Th2 undergo rapid apoptosis upon antigen-stimulation, and (2) Th2 clones preferentially survive in *in vitro* cell cultures, the progressive shift from type-1 to type-2 cytokine production

observed in HIV infection could be at least partially provoked by the increase in the production of cortisol and the reduction of DHEA (Clerici et al., 2000).

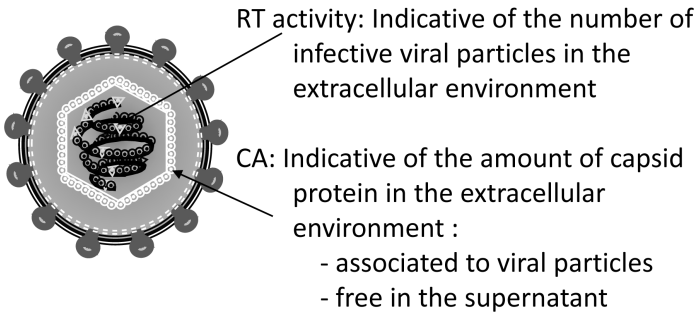

Figure 4. Different approaches for determining the effect of cortisol on the expression of viral particles.

In conclusion, it seems that GCs play a role in apoptosis. What consequences does this have for the retroviral cycle? Apoptosis (also called programmed cell death or PCD) is a type of cell death in which enzymes break down the cytoskeleton of the cell, upon which the cytoplasm becomes dense, with the organelles tightly packed. Characteristically, chromatin condenses and the nuclear envelope breaks, as well as the DNA (when run in a gel, a typical ladder of DNA fragments of different size may be seen). All this happens while the cell plasma membrane remains apparently intact, though its chemical composition is modified, and usually "blebs" are seen protruding from the cell. Lastly, the cell breaks down into vesicles, the apoptotic bodies. The modifications of the plasma membrane may have important consequences on the viral cycle of retroviruses, including FIV and FeLV.

Two parameters are especially useful to study the effect of cortisol on the viral cycle and whether apoptosis plays a role in this effect: RT activity and the amount of capsid protein in the extracellular environment. RT activity measures the presence of infective viral particles in the supernatant (Marozsan et al., 2004; Niermann and Buehring, 1997), under the assumption that only infective viral particles will have active RT. Unlike RT activity, the amount of capsid protein p27 of FeLV (FeLV-p27), and less markedly of p24 of FIV (FIV-p24), in the supernatant is not considered representative of the presence of viable particles (Figure 4). For unknown reasons, an undetermined percentage of the viral capsid proteins synthesized by some retrovirus is not incorporated into viable viral particles, and is released as free antigen or

defective particles (Rojko et al., 1996, 1992; Jarrett, 2001; Marozsan et al., 2004).

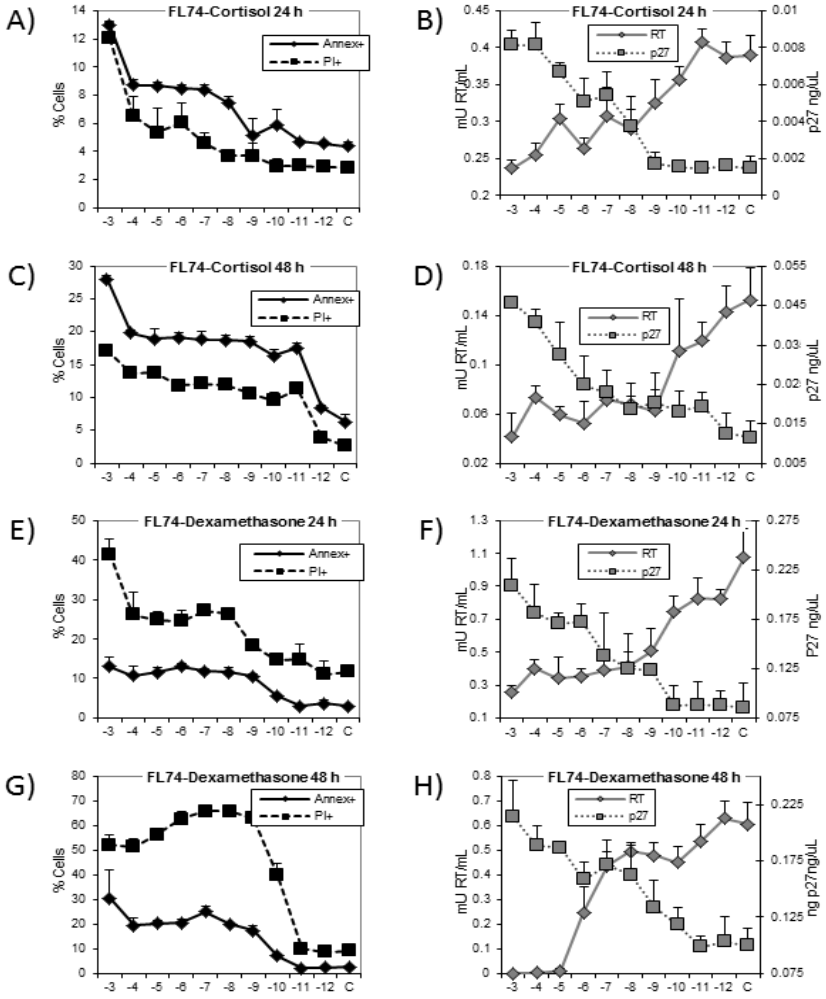

Figure 5. Effect of cortisol (A-D) and dexamethasone (E-H) on cellular viability and RT activity (mU/mL) and FeLV-p27 presence in the supernatant of treated chronically FeLV-infected cells (FL74) after 24 (A, B, E, F) or 48 (C, D, G, H) hours of exposure. Apoptotic cells are identified by the positive reaction to Annexin V (Annex+, PI-) while dead cells are identified by the positive reaction to PI (PI+). RT, RT activity.

EFFECT OF GLUCOCORTICOIDS ON FeLV-INFECTED CELLS

Interestingly, GCs had an opposite effect on FeLV-infected cells and on FIV-infected cells (Figures 5 and 6). In FeLV-infected cells, at high concentrations of both cortisol and dexamethasone, there was a high concentration of FeLV-p27 detected in the supernatant, which decreased as the hormone concentration decreased (Figure 5B, 5D, 5F, 5H). These high concentrations of FeLV-p27 could not be due only to cellular lysis (which would indeed promote the release of p27), as the results obtained when adding a lysis buffer were similar to controls with just culture medium. Thus, it could be assumed that cortisol and dexamethasone enhance the synthesis and release of FeLV-p27, independent from cellular viability, increasing its secretion. Contrariwise, the lowest RT activity values were invariably detected at the highest GCs concentrations (10^{-3}M) and at around 10^{-11}M they were comparable (in general) to that in the controls without hormone (Figure 5B, 5D, 5F, 5H). When cells were completely lysed using lysis buffer, high RT activity values were found in the supernatant, similar to controls, suggesting that simple lysis would free viable viral particles from cells. However, at 10^{-3}M of either cortisol or dexamethasone, even when cell counts and viability were very low (indicating high cell death), RT activity was considerably less than when using lysis buffer. This observation seems to suggest that in the presence of cortisol or dexamethasone cells might have died through a process of apoptosis, which might suppress the release of viable viral particles to the extracellular environment, possibly by alterations in the plasma membrane, thus presenting an inverse relationship between both variables (apoptosis and viral shedding). Taken together, these data may mean that high concentrations of GCs, such as cortisol or dexamethasone, enhance apoptosis of FeLV-infected cells. This would hamper the budding of infective particles (a parameter quantified by RT activity) and the increased protein synthesis would not culminate to form infective viral particles; thus, high concentrations of GCs would be protective for the cat. The effect of GCs on FeLV-infected cells agrees with results with 17β-estradiol and progesterone (Tejerizo et al., 2005).

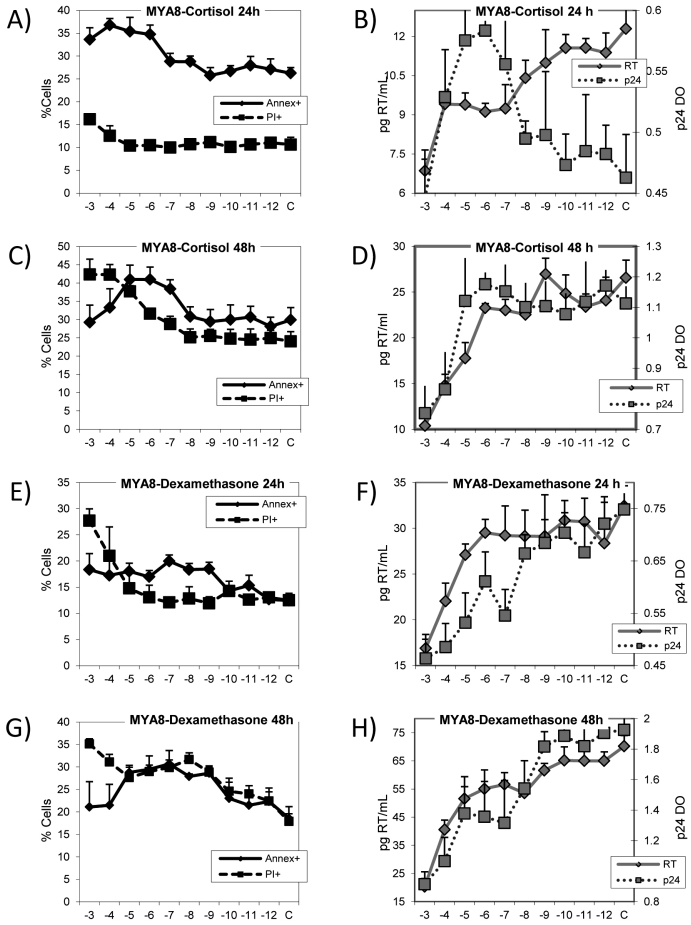

Figure 6. Effect of cortisol (A-D) and dexamethasone (E-H) on cellular viability and RT activity and FIV-p24 presence in the supernatant of treated FIV-infected cells (MYA-8) after 24 (A, B, E, F) or 48 (C, D, G, H) hours of exposure. Apoptotic cells are identified by the positive reaction to Annexin V (Annex+, PI-) while dead cells are identified by the positive reaction to PI (PI+). RT, RT activity.

EFFECT OF GLUCOCORTICOIDS ON FIV-INFECTED CELLS

Contrarily to what was observed in FeLV-infected cells, the expression of viral proteins in FIV-infected cells (as quantified by the presence of FIV-p24 in the supernatant) was repressed when these cells were exposed to high concentrations of either cortisol or dexamethasone, though RT activity levels

were similar in FeLV- and in FIV-infected cells (Figure 6). In the case of FIV, other additional mechanisms must exist that also influence RT activity, because the relationship between both parameters was not as homogeneous in FIV- as in FeLV-infected cultures and in some cases there was a decrease of RT activity without marked alterations in the rate of apoptosis. The greater biological complexity of FIV, mainly associated to the presence of regulatory proteins (encoded by *vif*, *rev* and *orf2*), could be responsible for the decrease of RT activity, somewhat independently from apoptosis.

Our findings do not agree with those reported in other studies. Barr et al. (2000) detected both an increase of the RT activity in PBMCs of prednisolone-treated FIV-infected cats, and an increase of plasma viremia in these same cats when compared to non-treated FIV-infected cats. An increase in the HIV RT activity was also found in cultures of lymphoid and monocytic cell lines incubated in the presence of cortisol and dexamethasone (Soudeyns et al., 1993). Niermann and Buehring (1997) also found an increase in RT activity of BLV (not a lentivirus, but also encoding a regulatory protein) in cultures of epithelial lung cells exposed to dexamethasone. According to these observations, it seems that GCs would favor viral replication and, therefore, the development of the infection, contrasting with our results, in which it appears that GCs inhibit the formation of infective viral particles. However, differences may be explained when analyzing assay conditions. For example, in the case of BLV, RT activity was studied in cultures selected for their resistance to GCs (Niermann and Buehring, 1997).

In previous studies with feline retroviruses (Barr et al., 2000), studies were done on cell populations isolated from infected animals treated with GCs, without the *in vitro* addition of GCs. In this case, other secondary factors derived from the immunosuppression induced by this hormone may play a relevant role.

It could be argued that the physiological concentration of cortisol in healthy cats is in the range of 10^{-8}M, and even in infected cats it is not higher than 10^{-7}M, concentrations far lower than those in which the most evident effects of hormones were observed. However, in the course of the experiments described here, it was also observed that the "freshness" of the cells affected the results, and cells which had been passaged 7-10 days before the beginning of the experiment at extreme conditions (1:40) were affected by the hormones at concentrations far lower (10^{-8}M) than cells passed 1:5 two or three days before the beginning of the experiment. It may be assumed that circulating lymphocytes can correspond to this "aged" population rather than to the "fresh" population, as renovation from the bone marrow does not happen as

fast as in culture media *in vitro*. Thus, circulating lymphocytes would be more affected by lower concentrations of hormones.

From the results presented above it became evident that GCs affected the presence of feline retroviruses in the extracellular environment. The effect was both through indirect and direct mechanisms. Indirectly, by activating cellular apoptosis, GCs may suppress budding of virions to the extracellular environment. This would benefit the cat, and is more obvious in the case of FeLV.

Glucocorticoids also had an effect on viral protein expression, which was the opposite in FeLV and in FIV. The increased expression of FeLV-p27 and decreased of FIV-p24 at high doses of cortisol or dexamethasone suggest that these GCs had a direct effect on viral expression, stimulating or repressing the transcriptional ability of these feline retroviruses. Together with the sharp increase in the serum levels of cortisol detected in infected cats, these data suggest an important role for GCs in the pathogenesis of FeLV and FIV.

EFFECT OF CORTISOL AT THE MOLECULAR LEVEL

Few studies have been undertaken to determine the effect of steroid hormones directly on the expression of feline retroviruses. The molecular mechanism of action of cortisol, as in the case of other steroids, involves binding to intracellular molecules. Cortisol is able to diffuse through the cell membrane into the cytoplasm, where it binds to the glucocorticoid receptor (GR). Homodimer GR-cortisol complexes are translocated to the nucleus, where they bind specific DNA sequences called glucocorticoid responsive elements (GRE) (or more broadly, hormone responsive elements or HRE) and activate gene transcription (Figure 7). A second mechanism involves the binding of GR-cortisol to NF-κB or AP-1 factors or to the DNA binding sites (called transcription binding sites or TBS) for them, to repress them from transactivating target genes. These two mechanisms are called transactivation and transrepression, respectively.

HRE are responsible for the biological effects of cortisol, which depend also on the type of cell exposed to the steroid. For example, when GR-cortisol complexes cross the nuclear membrane of Th cells, they bind GRE which transactivate the IL-4, IL-10 or IL-13 genes and differentiate the lymphocyte into a Th2 cell. The GRE sequence has been identified to be TGTTCT, and is common for glucocorticoids, progesterone, androgens and mineralcorticoids.

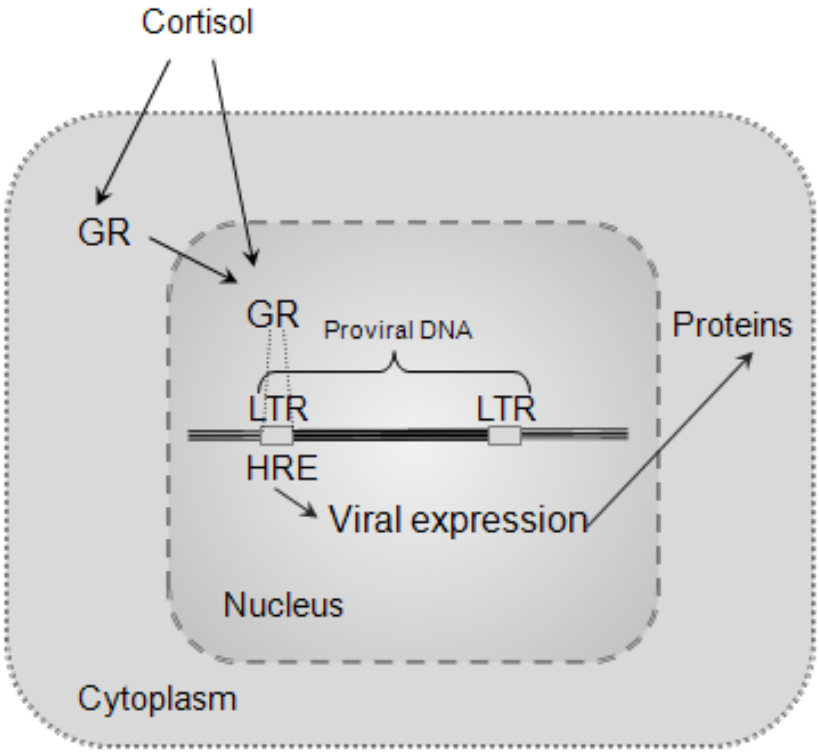

Figure 7. Direct mechanism of protein expression transactivation by cortisol. Due to its lipophilic nature, cortisol may freely cross cellular membranes to bind its receptors (GR), which are in the cytoplasm or in the nucleus. The complex cortisol-GR crosses the nuclear membrane and is recognized by specific DNA sequences called glucocorticoid responsive elements (GRE). GREs have been found in the LTR of several retroviruses. The recognition of the cortisol-GR complex by these GRE in the LTR triggers transcription which culminates in the expression of viral proteins.

The first indication that steroids could affect the expression of retroviruses stems from the 60s when Smoller et al. (1961) observed inclusion bodies in the cytoplasm of cortisol-treated mammary tumors produced by the mouse mammary tumor virus (MMTV).

It was found that when dexamethasone (a potent synthetic glucocorticoid), progesterone, testosterone or mineralcorticoids were added to the culture medium of cells infected by MMTV, there was an increase in the viral RNA (Ringold et al., 1975; Smoller et al., 1961), suggesting that GCs could stimulate replication and expression of the virus. Later, the presence of a GRE was identified in the genome of MMTV (Majors and Varmus, 1983; McGrath,

1971; Payvar et al., 1983; Slater et al., 1989; Stewart et al., 1988). This GRE is a sequence 75 to 195 base pair (bp) long mapped to the 5' long terminal repeats region (LTR, Figure 7). LTRs are sequences which flank the viral genome in the state of provirus (*i.e.* when it is integrated in the host DNA), playing crucial roles in transcription. The relationship between steroids and MMTV has been intensely studied, becoming one of the most important models for understanding genome regulation. The effect of steroids directing transcription has also been shown in other retroviruses such as Moloney murine sarcoma virus, murine endogenous retroviruses, bovine leukemia virus, porcine endogenous retroviruses, and simian and human immunodeficiency viruses (Diallo et al., 2000; Ghosh, 1992; Lan et al., 1984; Miksicek et al., 1986; Niermann and Buehring, 1997; Quinn and Langford, 2001; Ramakrishnan and Robins, 1997; Rousseau, 1984; Russo et al., 1999). Mutations in this site in several retroviruses induce the decrease of transcriptional activity when exposed to GCs, proving their role in transcription (Nishigaki et al., 1997). In FeLV, a GRE was located in the LTR (GRE: nt. -184 to -179) (Fulton et al., 1990; Nishigaki et al., 1997). To the best of our knowledge, no such element has been located in FIV. GCs-mediated regulation has also been demonstrated in DNA viruses, such as herpesvirus and papillomavirus (Poreba et al., 1998; Slater et al., 1989). However, data and studies related specifically to feline retroviruses are scarce.

In order to determine whether cortisol or dexamethasone would have an effect on FIV and FeLV transcriptional activity, their LTR was cloned into pEGFP-1 vector (Clontech) which does not contain any promoter of itself. Plasmids were transfected into CrFK cells and cells were treated with 10^{-3}M to 10^{-11}M of either cortisol or dexamethasone. Controls included cells transfected but not treated with glucocorticoid (C), and cells transfected with the empty plasmid (pEGFP). The expression of EGFP was quantified by flow cytometry 24 h after treating transfected cells with cortisol or dexamethasone.

In addition, EGFP allowed following the efficiency of transfection and the state of the cell population by fluorescence microscopy, locating and characterizing specifically cells based on their expression levels.

In the case of the plasmid containing the LTR of FeLV, higher expression of EGFP was observed at the higher concentrations of both cortisol and dexamethasone (Figure 8A and 8B), while in the case of the plasmid containing the LTR of FIV, the opposite was observed (Figure 8C and 8D). This parallels the findings of the quantification of the capsid protein in the supernatant upon the exposure to the glucocorticoids.

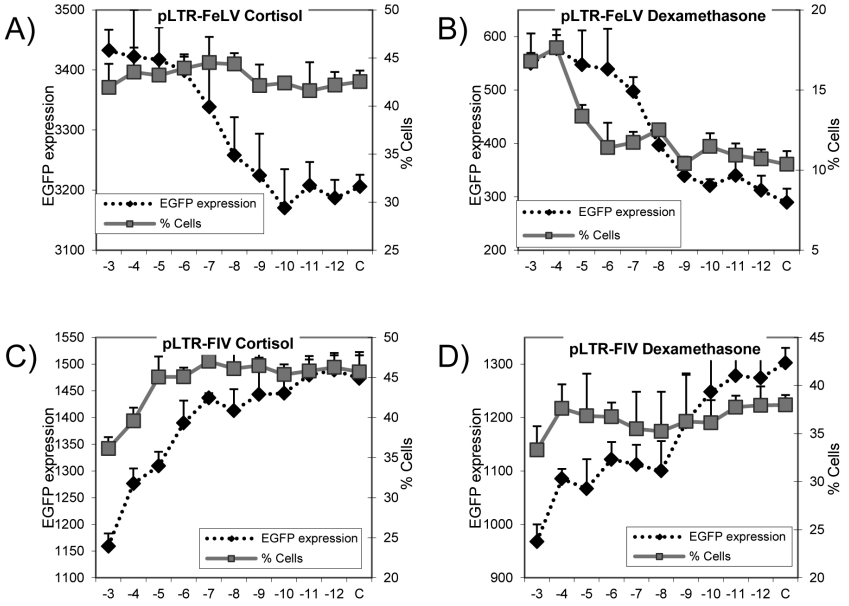

Figure 8. Effect of cortisol (A, C) and dexamethasone (B, D) on the expression and on the percentage of cells expression EGFP in CrFK cultures transfected with a plasmid containing the LTR of FeLV (Rickard A strain) (A, B) or the LTR of FIV (Glasgow 8 strain) (C, D) after 24 hours of incubation.

We may conclude that both FIV and FeLV seem to have a functional GRE, as the expression of EGFP varies with the different concentration of cortisol or dexamethasone.

Our data on FeLV increased expression of EGFP and FeLV-p27 at high concentrations of GCs associated to the GRE sequence present in the LTR coincide with those obtained by Nishigaki et al. (1997). These researchers showed that mutations in the LTR-FeLV GRE region led to a decline in the transcriptional activity in both feline and human lymphoid cell lines and in CrFK fibroblasts. In addition, this region is highly conserved in different clones of FeLV (Fulton et al., 1990), which suggests its probable involvement in viral regulation.

As the expression of FeLV viral proteins could increase in the presence of GCs, and cortisol concentration is significantly increased in FeLV-infected cats, viral genomes which take advantage of this could have been selected along the evolution to maximize their transmission and to escape the immune response. The observations of Rojko et al. (1979) would support this hypothesis. They showed that FeLV-infected cats treated with prednisolone

lost their natural resistance to persistent viremia, *i.e.* treatment with GCs favored the survival of the virus.

On the other hand, the response to cortisol via LTR could play an additional role in the development of tumor processes, strongly associated with oncogenic viruses such as FeLV, by the insertional activation of different protooncogenes (Dudley et al., 2002; Morrison et al., 1995). The proviral LTR sequence carrying a functional GRE could be inserted close to oncogenes, providing them with certain degree of sensitivity to hormonal activation (Beato et al., 1989). An increase of GCs, associated with factors such as stress or pregnancy, could thus trigger an adjacent protooncogene, leading to its expression and ultimately to the abnormal growth of the cell and tumorigenic processes.

Contrarily to FeLV, transcriptional activity directed by the FIV-LTR was suppressed at high GC concentrations, coinciding with the results of the detection of FIV capsid protein (FIV-p24) in the supernatant. In the case of HIV-1, a GRE site has been found in its *vif* sequence, which favors viral replication when exposed to GCs (Soudeyns et al., 1993). In FIV, which is also a lentivirus, the existence of this GRE site or any other GC binding consensus sequence in the viral LTR has not been described. Data shown above suggest that the effect of cortisol would be transrepression as explained at the beginning of this section, instead of transactivation, not involving a GRE but rather other cellular pathways such as NF-κB or AP-1 as described below.

Despite a growing body of evidence on the relationships between pathogens and GCs, little is known yet on the effect of these steroids on viruses associated with immunodeficiency syndromes. A regulatory effect of the GCs on different retroviruses has been reported. The results obtained for lentiviruses, mainly HIV, are heterogeneous. However, they all agree in accepting the involvement of GCs in its pathogenesis. GCs have been described to both activate and repress the expression of HIV, and some researchers have shown the presence of several GREs in its sequence, both in the LTR and in intragenic regions (Ghosh, 1992; Kino et al., 2000; Mitra et al., 1995; Russo et al., 1999; Soudeyns et al., 1993; Vanitharani et al., 2001). In general, it seems clear the possibility of a differential regulation depending on the cell type and the intracellular factors specific for each cellular lineage (Kino et al., 2000; Mitra et al., 1995).

It is becoming increasingly apparent that the mechanisms of action of the GCs are more varied from what until recently has been believed. Currently, intracellular processes based on the direct regulation of transcription (specific binding of the GC-GR or GR, to TBS), in addition to the classic mechanism,

are recognized. These processes do not involve the complex GC-GR, although it is believed that they do require the glucocorticoid receptor (GR), and involve a number of interactions with different biochemical routes.

Our results show that GCs inhibit transcription directed by the FIV-LTR in lymphoid lines and fibroblasts. These data coincide with those published for HIV by other researchers (Kino et al., 2000; Mitra et al., 1995). In these cases, the GCs effect appears to be mediated by GREs located in different positions within the LTR, mainly at the site +15 to +20 (Mitra et al., 1995), but there is no agreement about the functionality of the remaining sites. The LTR region of FIV does not present the GRE consensus sequence, which challenges an explanation based on this interaction between the hormone and DNA. GCs could interact, either with yet unidentified similar viral sequences, and regulate replication by a direct mechanism, or with other cellular factors involved in the regulation of viral replication by an indirect mechanism.

The *direct mechanism* to explain the decreased transcriptional activity of the FIV-LTR in the presence of high GCs concentrations would involve the existence of sequences similar to a GRE overlapping a positive regulatory factor. The union of GR to this site would displace the binding of this second factor, thus interfering with its activating effect. Different DNA sequences could support this hypothesis (Figure 10B), such as the TTGACT sequence (nt -125 /-120), which overlaps with the AP-1 transcription factor binding site (nt -124/-118). Something similar could happen in the sequences TGTTTT (nt -51/-46) and TGTTCC (nt -46/-41), due to their proximity to the ATF transcription factors binding sites (nt -58/-53).

Mechanisms involving other cellular signaling molecules (*indirect regulation*) are currently considered to explain the transrepression observed when protein expression was decreased when FIV-infected cells (cellular experiments) or FIV LTR (molecular experiments) were exposed to increased concentrations of GCs.

AP-1 and ATF are important regions in the regulation of FIV and are involved in the basal promoter activity of the LTR (Chatterji et al., 2002; Ikeda et al., 1998; Inoshima et al., 1996; Murphy et al., 2014; de Parseval and Elder, 1999; Thompson et al., 1994). Ikeda et al. (1998) showed that the nuclear factors that bind specifically to AP-1 and ATF sites of the FIV-LTR are present in several cell lines, including the two used in our experiments (CrFK and MYA-1). However, while these factors have similar properties, they are not exactly the same in both lines. Deletion experiments showed that the promoter activity was reduced in the absence of any of these fragments (AP-1 or ATF); this decrease was even more evident in the case of a double

deletion, suggesting cooperation in the mechanism of transcriptional regulation. Similar results were obtained with deletion mutants in sites AP-1, C/EBP and ATF in the FIV-LTR in the CrFK cell line (de Parseval and Elder, 1999). On the other hand, in the case of AP-1, this assumed competition with the hormone-receptor complex would not be decisive for the replication of the virus, as other studies have proven that its deletion does not completely inactivate the process (Miyazawa et al., 1993), although it could be slower (Inoshima et al., 1996). In the case of FeLV, AP-1 (which has four sites not represented in the FeLV-LTR, Figure 9A) has been involved in the opposite phenomenon as in FIV: transactivation. Activation of mitogen-activated protein kinases 1 and 2 (MEK1 and -2) by the LTR is an intermediate step in the FeLV LTR-mediated induction of AP-1 activity (Ghosh and Faller, 1999). This would agree with our results.

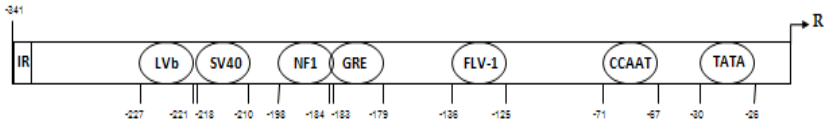

Figure 9. Schematic representation of the FeLV genome showing the most relevant transcription binding sites and their relative location.

It has also been shown that the AP-4 and AP-1 sites, which are quite close together (Figure 10A), can activate regulatory regions in a coordinated way (Mermod et al., 1988). This could be the case for the AP-4 (nt -134/-129) and AP-1 sites (nt -124/-118) of the FIV-LTR (de Parseval and Elder, 1999), where the efficient binding with their transcription factors could be affected by the presence of the GC-GR complex in a hypothetic adjacent GRE sequence. Lastly, and further supporting the importance of AP-1 and ATF sites, it is believed that the assumed transactivating Tat protein of FIV (ORF2) interacts with these sites of the LTR (de Parseval and Elder, 1999). The GC-GR complex could interfere with this interaction also by this mechanism.

Other proteins which have been thought to play a role in the repression of protein expression by GCs in retrovirus-infected cells are STAT (signal transducer and activator of transcription) 5, IκBα, nuclear factor I (NFI), and CCAAT displacement protein (CDP). STAT5 is activated when cells are exposed to IL-2 and GCs, inhibiting the GR transcriptional activity (Biola et al., 2001). In the case of IκBα, GC treatment increases the levels of this protein and reduces the amount of NF-κB, a strong activator of HIV-1 promoter, decreasing by this mechanism the transcription of HIV-1 (Kurata

and Yamamoto, 1999). NFI sites have been shown to compete with the GC receptors for DNA binding, decreasing by this mechanism the effect of GCs in the MMTV model (Brüggemeier et al., 1990). This mechanism would be similar to that proposed by Zhu and Dudley (2002), in which CDP, expressed in high amounts in the naive mammary gland, would repress MMTV expression, playing a role in tumorigenesis.

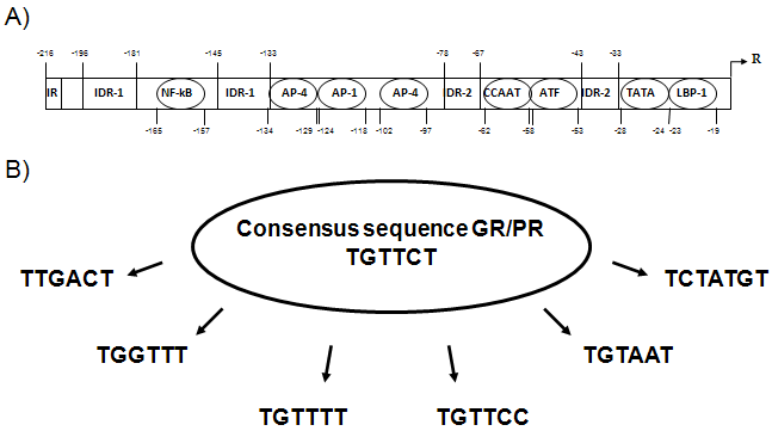

Figure 10. Transcription binding sites (TBS) in the LTR of FIV which could explain the transrepression observed when FIV-infected cells or FIV-LTR are exposed to high concentrations of GCs. A) Schematic representation of the FIV genome showing the most relevant TBS and their relative location. B) Alternative GRE sites in the FIV LTR. The consensus sequence for the glucocorticoids and progesterone (GR/PR) is shown in the oval.

Besides the effect through signaling molecules, other mechanisms of repression may be possible. For example, in HIV-1 LTR-independent pathways influenced by cytokine and GC through which HIV can maintain substantial levels of protein expression and virion production have been proposed (Kinter et al., 2001). These may involve Vpr (Mirani et al., 2002), which modulates host GR function to affect transcription of host genes, probably acting as a co-activator for the GR (Hapgood and Tomasicchio, 2010). Different isoforms of the glucocorticoid receptor have been recently described. Differential post-transcriptional processing and modification may give rise to different variants of this GR. It has been suggested that this variety of isoforms could lead to unique biological responses (Zhou and Cidlowski, 2005).

Although the differences between these isoforms are still greatly unknown, the presence of isoforms of GR in certain cell types may be involved in negative responses.

In summary, the results obtained and data reviewed support that glucocorticoids are important factors in the regulation of FeLV and FIV, through direct mechanisms, indirect mechanisms or, possibly, by the coordinated action of both. The increased plasma concentration of cortisol in FeLV-infected cats and the effect that high concentrations of cortisol have, increasing viral protein expression, should be taken into consideration when considering treating cats with immunosuppresants to treat FeLV-associated clinical signs. The role of glucocorticoids in feline retroviral infections is an exciting field which requires more studies.

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