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A study of the process of apoptosis in animals infected with the contagious ecthyma virus

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Abstract

Contagious ecthyma virus (CEV) is a disease caused by a parapoxvirus, also is a potent genetic carrier with the capacity for regulating apoptosis in the cells of infected skin, a mechanism that serves for evading the immune response of the host. It has been suggested that the virus may remain in the skin and be able to cause repeated infections in the same flock.

The effect of infection as well as the presence of contagious ecthyma virus was evaluated in terms of lesions and apoptosis in the skin of animals, infected both naturally and experimentally.

Samples used were obtained from a naturally infected sheep, 5 goats inoculated with CEV and a negative control. Samples obtained were longitudinally sectioned and processed using photon and electron microscopy, and embedded in paraffin and araldite. Samples embedded in paraffin were sectioned in 5 μm of thickness and dyed with orange eosin–hematoxilin G and Gomori's trichrom stain, apoptosis was demonstrated by the TUNEL assay, the viral antigen was revealed using polyclonal antibodies, and the presence of lymphocytes CD4+ and CD8+, with monoclonal antibodies. The samples processed in resin were cut to obtain semi-fine sections and dyed with toluidine blue-borax, and the ultra-fine sections were impregnated with lead citrate and uranyl acetate.

Observations were similar in both, the natural infected animal and the experimental group. Infiltration was observed as well as images suggestive of a process of apoptosis. The TUNEL assay demonstrated that the number of epithelial cells undergoing apoptosis diminished during the process and increased among defense cells, until they almost disappeared at the beginning of healing. Cells undergoing apoptosis were located near the sebaceous glands and pilose follicles. The infiltrated lymphocytes gradually diminished. The viral antigen was observed in cells with morphology suggestive of apoptosis, located in sebaceous

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glands and pilose follicles. Using electron microscopy, cells with morphology compatible with that of lymphocytes were observed to be undergoing apoptosis, but there was little evidence of viral particles.

Keywords: Apoptosis; Contagious ecthyma; TUNEL assay; CD4+; CD8+

1. Introduction

Contagious ecthyma is a disease caused by a particular parapoxvirus, which is characteristic during its severest phase produce lesions in progression from papules to pustules and then to thick scabs or crust, the pustulating lesions affect the mucocutaneous borders and mucous membranes of sheep and goats. The contagious ecthyma virus (CEV) causes a debilitating disease of high morbidity 80%, and low mortality 5–10%, which occasionally affects humans (Fields et al., 1996; Haig and Mercer, 1998; Tórtora et al., 1998; Mercer and Haig, 1999; Regenmortel van et al., 2000).

Young animals are particularly susceptible, suffering from lesions which commonly appear around the mouth and nostrils. The lesions may reduce their ability to feed themselves, to suckle and graze, and secondary infections may also appear along with bacteria, fungi or insect larvae (Haig et al., 1999). Ecthyma may become a serious problem, as it can provoke mortality in animals under stressful conditions and immune suppression among overcrowded sheep or animals transported over long distances (Haig and Fleming, 1999).

Certain aspects of the disease have still not been clarified, the virus can infect sheep and goats repeatedly, in spite of the fact that the host apparently presents a vigorous immune response and inflammation (Haig et al., 1996, 1997a,b). Another aspect which remains to be clarified is that among animals with skin infection, there is no evidence of systemic infection.

The virus is found in the material originating from scabs and it has been suggested that outbreaks occur because of contact with infectious viruses in the environment, or it is also possible that the virus maintains itself in the flock at sub-clinical levels, causing periodical undetectable damage to the skin, although this theory has not been rigorously explored (Haig and Mercer, 1997). There is however evidence of viral transmission between clinically healthy sheep and sheep that have not had contact with the virus (Nettleton et al., 1996).

The immune response against infection by CEV in skin and local lymph nodes, presents the characteristics of an antiviral reaction including CD4+, CD8+ cells, cytotoxic T lymphocytes, interferons, antibodies and other complementary components (Haig et al., 1998; Deane et al., 2000). Viral antigens have been identified which regulate the immunity of the host (Gooding, 1992), as well as genes which are related to virulence such as: a gene homolog for the vascular endothelial growth factor (VEGF) (Lyttle et al., 1994), a homolog of sheep interleukin-10 (IL-10) (Fleming et al., 1997) and a gene resistant to interferon (E3L) which is a homolog of the vaccinia virus gene (Chang et al., 1992).

Viral activity inhibits the granulocyte-macrophage colony-stimulating factor which may be responsible for temporary viral elusion, thus allowing replication in cells (Alcami and Smith, 1995; Haig et al., 1998; Alcami and Koszinowski, 2000; Deane et al., 2000). Among viral virulence mechanisms, the modulation of the apoptotic process and the genetic transmission of cells infected by the poxvirus may be considered (Haig et al., 1998; Turner and Moyer, 1998).

Apoptosis consists of the programmed death of cells, which permits the selective and rapid elimination of proliferating cell populations, a process which is observed during embryonic development, morphogenesis and metamorphosis, as well as in the atrophy of endocrine tissue, in the normal change and transformation of tissues, in the removal of undesirable cells during recovery from inflammation and during neoplastic regression (Aderem and Underhill, 1999; Cotter, 1990). Programmed cell death also plays a decisive role in the development and maturation of T lymphocytes (Rubin and Kretz-Rommel, 1999), the elimination of lymphocytes which recognize the self in the thymus gland, it preserves peripheral homeostasis and promotes tolerance towards T cells

stimulated by environmental antigens (Cohen, 1991; Lenardo et al., 1999; Abbas and Lichtman, 2000) and plays an important role in the regulation of Th1 and Th2 cells (Morel and Oris, 1998).

The skin is a very dynamic organ and keratinocytes are constantly replaced at the level of epidermis. These proliferate, differentiate and migrate towards the surface. In order that this tissue maintains its homeostasis, a process of programmed cell death is necessary (Van Laethem et al., 2005; Zuliani et al., 2005). In this context, CEV may be able to adequately modulate the repression or promotion of apoptosis by producing specific proteins, as well as controlling whether it remains in infected tissue cells even within the individual.

In this study, the effects of infection as well as the presence of particles suggestive of CEV on the skin and in lesions of infected animals were studied, using bright field microscopy (BFM) and transmission electron microscopy (TEM). The TUNEL assay was used to identify cells undergoing apoptosis and their relationship to CEV was established, the presence of CD4+ and CD8+ cells was determined and viral antigens were immunolocalized.

Our results indicated the presence of particles suggestive of CEV in cells with the morphology of lymphocytes, which presented different stages of apoptosis. In the presence of particles suggestive of CEV, apoptosis diminished in keratinocytes and increased among defense cells. A relationship was observed of CD4+ and CD8+ cells with the progenitor cells of sebaceous glands and pilose follicles, which were also positive to polyclonal antibodies against viral antigens.

2. Material and methods

2.1. Virus

For the purposes of experimental infection, viruses were isolated from scabs obtained from a merino sheep suffering from a natural outbreak. The scabs were soaked in minimal essential medium (MEM) used for cell culture, along with 2% of penicillin–streptomycin and centrifuged at 1200 × *g* for 10 min at room temperature (Tórtora and García, 1987). Later, these were re-suspended and concentrated on a 30% saccharose cushion in PBS (García-Tovar, 1993; Mahy and Kangro, 1996).

The polyclonal serum was obtained using lyophilized virus at a titer of up to 10^{-8} units CPE in BHK-21 clone 13 cells, per ml. The lyophilized virus was prepared by collecting scabs of goats which were macerated in MEM, along with 2% of penicillin–streptomycin and then centrifuged at 1200 × *g* for 10 min at room temperature using peptone saccharose as a lyophilizing medium, and was then kept at 4 °C until use (Tórtora and García, 1987). In order to use the lyophilized virus, it was re-suspended and concentrated on a 30% saccharose cushion in PBS (García-Tovar, 1993; Mahy and Kangro, 1996).

The viability of both viruses was measured by observing the viral concentrates with transmission electron microscopy under negative staining in order to identify the type M viral particles or any infectious particles.

2.2. Anti-CEV polyclonal serum

Anti-CEV polyclonal serum was obtained inoculating 2 male rabbits weighing between 2 and 3 kg. Viral lyophilizate was concentrated by means of ultracentrifugation on 30% saccharose (García-Tovar, 1993; Mahy and Kangro, 1996). Finally, complete Freund adjuvant and Melox^{MR} were also added (Bautista and Morilla, 1989; Hudson and Hay, 1992).

2.3. Animals

Animals of 6 months of age were used (±20 days): 6 goats of which 5 were inoculated, and 1 which was used as a negative control, as well as 1 naturally infected sheep which was used as positive control. Animals were kept in metabolic cages and fed with concentrated commercial alfalfa hay with water *ad libitum*.

Treatment: skin samples were obtained from the positive control (naturally infected) and were representative of the 3 zones which are characteristic of lesion evolution: erythema, vesicular and humid scabs.

Goats were inoculated by scarification in the previously shaved lumbar region. Samples were obtained from a wound, starting at 6 h post inoculation (PI) and then at 12, 18 and 24 h PI, and at 2, 3, 4, 5, 6, 7, 8 and 9 days PI. The skin was repaired with cross-stitch.

Among CEV-inoculated animals, a control was performed by scarring the left hand side of the lumber region with phosphate buffer solution (PBS). A second control was performed using a clinically healthy animal, which was scarred using the same procedure, applying PBS on the right hand side, and only scarifying the left side.

2.4. Sample processing

Samples were selected transversally with respect to the line of inoculation and/or scarification, obtaining two sections (cranial and caudal). The first sample (cranial half) was processed for transmission electron microscopy and fixed using Karnovsky's mixture, post fixed with osmium tetroxide and then processed for araldite resin embedding (Glauert, 1991). Sections of 100–120 nm were obtained and impregnated with lead citrate and uranyl acetate (Glauert, 1991) to be observed by TEM. The second sample (caudal half) was processed for photon microscopy, fixed in paraformaldehyde at 2.5%, at 4 °C. It was routinely embedded in low melting point paraffin and sections of 5 μm of thickness were obtained (Bancroft et al., 1999), which were then stained with eosin–hematoxilin and Gomori's trichromic technique (Conn and Darow, 1944; Lillie, 1954). The semi-fine sections (1–2 μm) obtained from the pieces embedded in araldite were dyed with toluidine blue-borax (Glauert, 1991). Scabs caused by both natural and experimental infections were examined by negative staining.

3. TUNEL assay

In situ cellular death was detected by histochemical analysis with the ''In situ Cell Death Detection Kit, AP'', by Boehringer-Mannheim, which uses DNA fragments marked with fluorescein (Catalogue 1 684 809) (Cuello-Carrion and Ciocca, 1999; Fertig et al., 1998, Kang et al., 2000; Timm and Carleton, 1996; Wyllie et al., 1998).

3.1. Immunohistochemistry

Rabbit polyclonal anti-CEV was applied at a concentration of 1:5 on paraffin sections. A commercial peroxide-marked mouse anti-rabbit antibody (FITC # F4890 Sigma) was used at a concentration of 1:50 to reveal CEV proteins (Bancroft et al., 1990; Ternyinck and Avrameas, 1989).

CD4+ and CD8+ lymphocytes were located by means of a DAKO LSA B+ System HRP (code K0679 CA, USA) commercial kit, demonstrating the presence of the antibody using diaminobenzidine.

3.2. Statistics

The tables show the average and standard deviation of 6 fields counted. Significant differences between multiple groups were analyzed by ANOVA, followed by one factor variance analysis ($p = 0.05$).

4. Results

4.1. Bright field and transmission electron microscopy

No morphological differences were observed between the lesions produced by natural outbreak and those produced by experimental infection. Generalized infiltration of mononuclear cells and groups of mononuclear cells were observed in the wounds near the basal membrane of the dermis (Fig. 1b and c). Cells with fragmented nuclei suggestive of apoptotic process were observed at the vesicular base using bright field microscopy (Fig. 1d and e). Inclusion bodies were observed in the macular, papular and vesicular stages, which disappeared during the healing of lesions (Fig. 1b). Cells matching the morphology of monocytes, macrophages and lymphocytes were observed under electron microscopy. These cells presented different levels of nuclear fragmentation, suggesting different stages of apoptosis inclusions and structures corresponding to the size of the CEV were observed, as well as areas of viral genesis, as reported for other viral infections (Fig. 2). The scabs caused by both natural and experimental infections, exhibited viral particles characteristics of CEV.

4.2. Locating apoptosis by means of the TUNEL assay

A great number of cells with the same morphology as that of monocytes, macrophages and lymphocytes

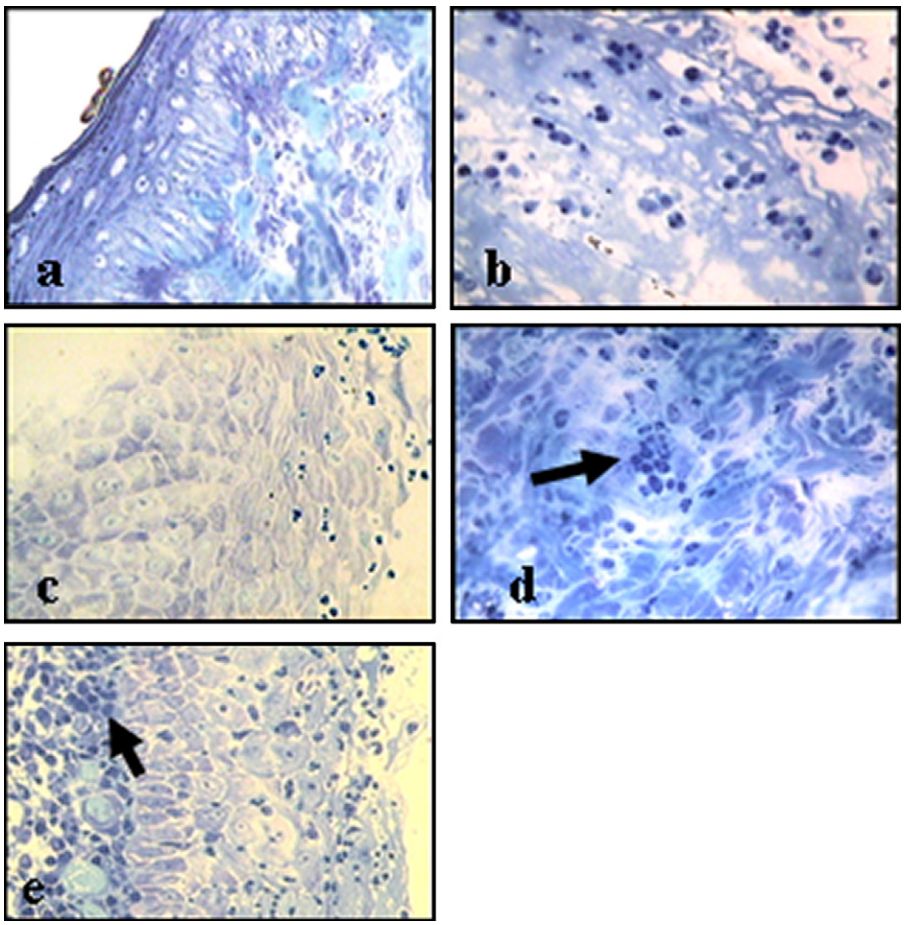

Fig. 1. Histology of normal skin and of skin infected with CEV. (a) Normal goat skin sample, semi-fine section, toluidine blue-borax, 40×. (b) Sheep skin sample with natural infection in vesicle stage, showing bodies suggestive of apoptotic cells (arrow head). Mononuclear infiltration of the dermis (black arrow), semi-fine section, toluidine blue-borax, 100×. (c) Sample of goat skin infected artificially, in vesicle stage sowing bodies suggestive of apoptotic cells (arrow head). Mononuclear infiltration of the subdermis (black arrow). (d) Sample of goat skin infected artificially, scab stage, showing cellular groupings in the subdermis (black arrow), mononuclear infiltration (white arrow) and bodies suggestive of apoptotic cells (arrow head). Semi-fine section, toluidine blue-borax, 100×. (e) Sample of sheep skin with natural infection, scab stage, showing cellular groupings in the subdermis (black arrow). Semi-fine section, toluidine blue-borax, 100×.

in the subepidermis were observed to be positive to the TUNEL. The number of apoptotic positive cells in the macular and papular stages was greater than that in the vesicular stage and almost disappeared in the healing stages (Fig. 3, Table 1).

4.3. Immunohistochemistry

The polyclonal antibodies identified viral antigen in mononuclear cells that also presented a morphology suggestive of apoptosis, principally in the hypodermis and in the progenitor cells of sebaceous glands and pilose follicles. The viral antigen was identified during the macular stage and gradually increased in the papular and vesicular stage. During the healing process the identifying mark was concentrated in sebaceous glands and pilose follicles (Fig. 4).

4.4. Immunocytochemistry CD4+–CD8+

Among healthy animals, no cells marked with CD4+ or CD8+ antibodies were observed on the skin.

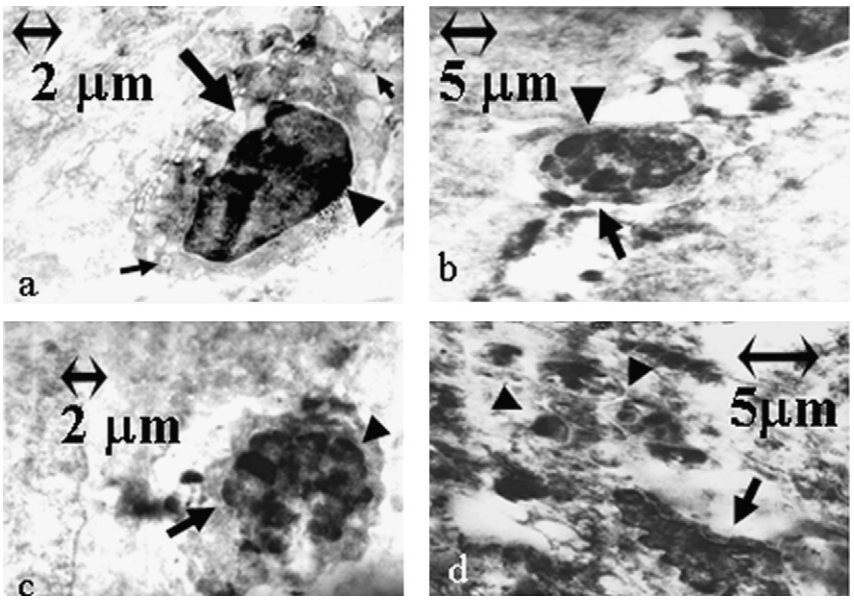

Fig. 2. Ultrastructural histology of cells at different apoptosis stages and of figures suggestive of viral particles. (a) Cells showing the beginning of chromatin condensation (arrow head) and contraction of the nuclear membrane (arrow). Structures are present that coincide with the shape and size of viral particles (small arrows). (b) Cell showing chromatin condensation towards the nuclear periphery (arrow head) and contraction at different places of the nuclear membrane (arrow). (c) Cell showing the start of individualization of the apoptotic bodies (arrow) as well as the complete condensation of the genetic material (arrow head). (d) Apoptotic bodies (arrow head) in relation with a keratinocyte (arrow).

Among infected animals, cells marked with CD4+–CD8+ antibodies were observed in the hypodermis from the macular stage onwards, their proportion increasing in the papular and vesicular stage and diminishing during healing. During the final stage of the process, cells marked with the CD4+–CD8+ antibodies were not observed in the external part of the dermis where the cells undergoing apoptosis were found. However, cells marked with CD4–CD8 antibodies were observed near to the pilose follicles and the sebaceous glands (Fig. 5, Table 2).

5. Discussion

The papapoxviruses infect a wide range of species, disease in sheep, goat, cattle and camels can be of economic significance, also infect several species of terrestrial and marine wildlife. These viruses are zoonotic, the human develop localized lesions.

Tórtora et al. (1998) described the morphological characteristics of cells, either infected or related to the process of infection by CEV. This description being suggestive a process of apoptosis. The present study proves the presence of cells in the process of apoptosis and describes their location with respect to the cutaneous and subcutaneous strata, sebaceous glands and pilose follicles in CE lesions.

It was not possible to locate viral particles by TEM suggesting their presence in the horny layer of samples which come from humid scabs, once healing had initiated. The morphology of the immune cells in apoptosis observed by TEM and BFM is characteristic of the process (Cotter, 1990). Mononuclear immune cells were observed to be undergoing apoptosis principally in the stages of papular and humid scab, which suggests that viral modulation occurs in defense cells, which may be induced by the virus in order to begin their own process of cell death, thus diminishing the immune response or inflammation of the host.

As in other pathological processes, cellular groupings characteristic of inflammatory infiltration were observed in the humid scab stage. Nevertheless, the virus is inhibiting the response of the host to the infection (recognition, presentation as well as cellular and humoral activity) and shows its capacity to

promote apoptosis through infected cells, which express themselves as promoters of the process, by diminishing the number and activity of defense cells.

The quantity of epidermal cells located by the TUNEL assay was significantly higher among healthy animals than among animals infected either naturally or artificially, and very similar in these two last cases, which suggests that inhibition of apoptosis occurs in the epidermis. Cells positive to the TUNEL assay were found in the subdermis and in the connective tissue relating to pilose follicles and sebaceous glands. This location coincides with that of CD4+ and CD8+ cells. The CD4+ and CD8+ cells located in the subcutaneous tissue diminished in number during the process of

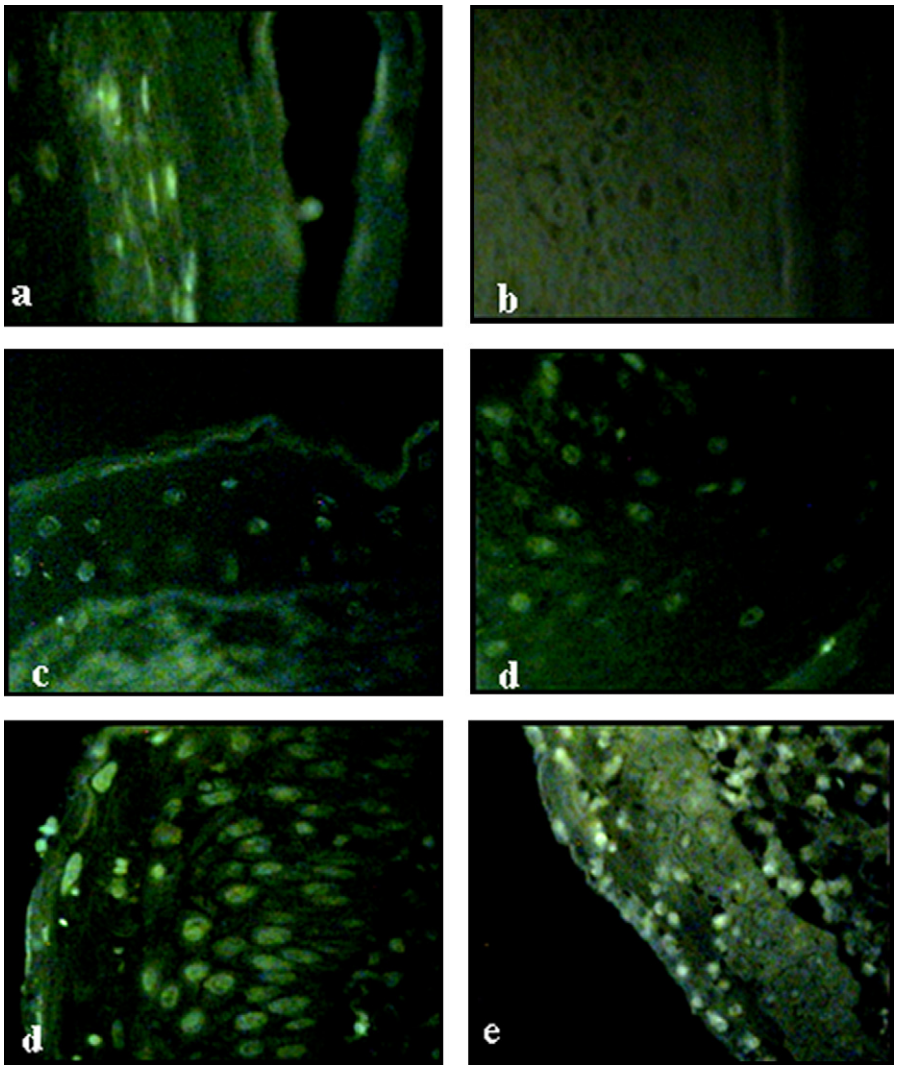

Fig. 3. (a) Skin section of control goat showing the localization and normal morphology of skin cells during apoptosis. (b) Skin section of control goat for the TUNEL assay. (c) Skin section of naturally infected sheep displaying the localization and morphology of apoptotic cells in infected skin during the macula stage. (d) Skin sample of artificially infected goat showing the peak of TUNEL positive mark at the papula stage. (e) Skin sample of artificially infected goat showing a decrease of the TUNEL positive mark at the vesicular stage. (f) Skin sample of naturally infected sheep showing a significant decrease of the TUNEL positive mark at the vesicular stage. (g) Skin sample of artificially infected goat showing the localization of the TUNEL positive cells in the subcutaneous tissue by contrast phase microscopy, during the scab phase. (h) Skin sample of artificially infected goat showing the localization of the TUNEL positive cells in the subcutaneous tissue, during the scab phase.

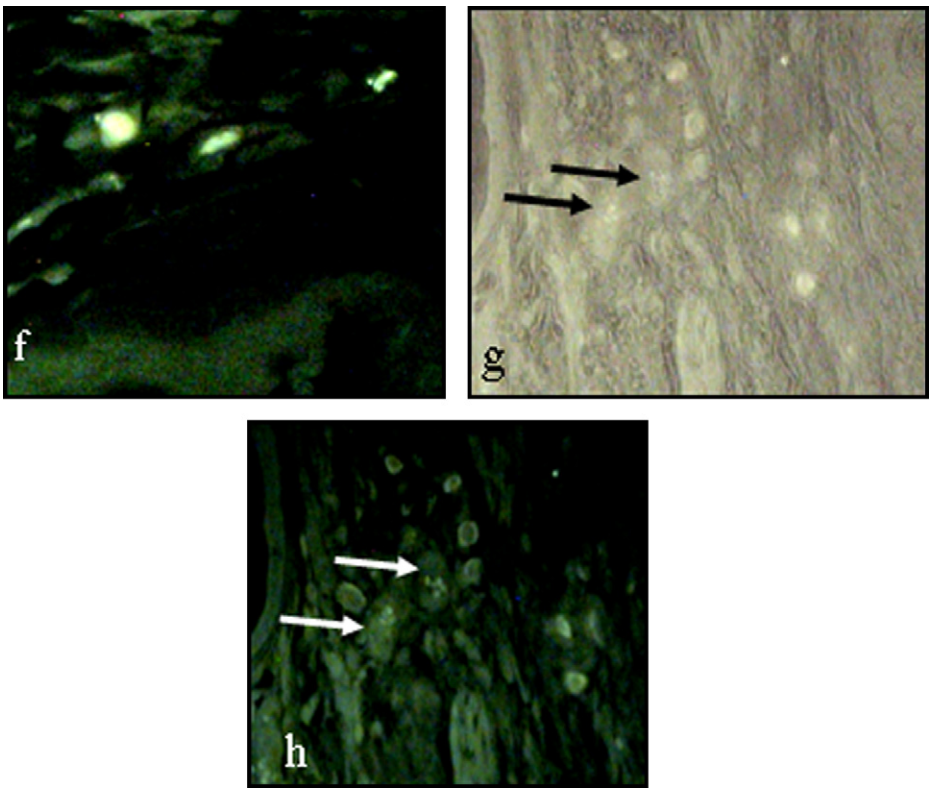

Fig. 3. (*Continued*).

Table 1
Number of fluorescent cells, in artificially infected goat skin with CEV and skin of naturally infected sheep with CEV, on TUNEL assay (TdT-dUTP-Nik End Labeling)

Animals	Days post inoculation		
	Day 5[a]	Day 7[a]	Day 9[a]
Epidermis in the inoculation zone data of 3 infected goats	10.5/3.7[b]	29.4/10.1	0
Dermis in the inoculation zone data of 3 infected goats	5.6/2.3[c]	12.1/4.2	8.7/3.3
Normal epidermis of 3 infected goats	5.6/1.2	5.1/1.4	4.6/1.2
Normal dermis of 3 infected goats	0	0	0
Epidermis of one normal goat	5.3/1.4[b]	–/–	–/–
Dermis of one normal goat	–/–	–/–	–/–
Epidermis whit lesions of a natural infected sheep	7.6/3.2	37.8/6.7	–/–
Dermis whit lesions of a natural infected sheep	0.6/0.8	10.1/2.9	13.1/3.1
Epidermis of a natural infected sheep whit out lesions	11.5/2.4[c]	–/–	–/–
Dermis of a natural infected sheep whit out lesions	1.6/1.2	–/–	–/–

6 fields were counted.
[a] Average/standard deviation.
[b] Significantly different.
[c] Significantly different.

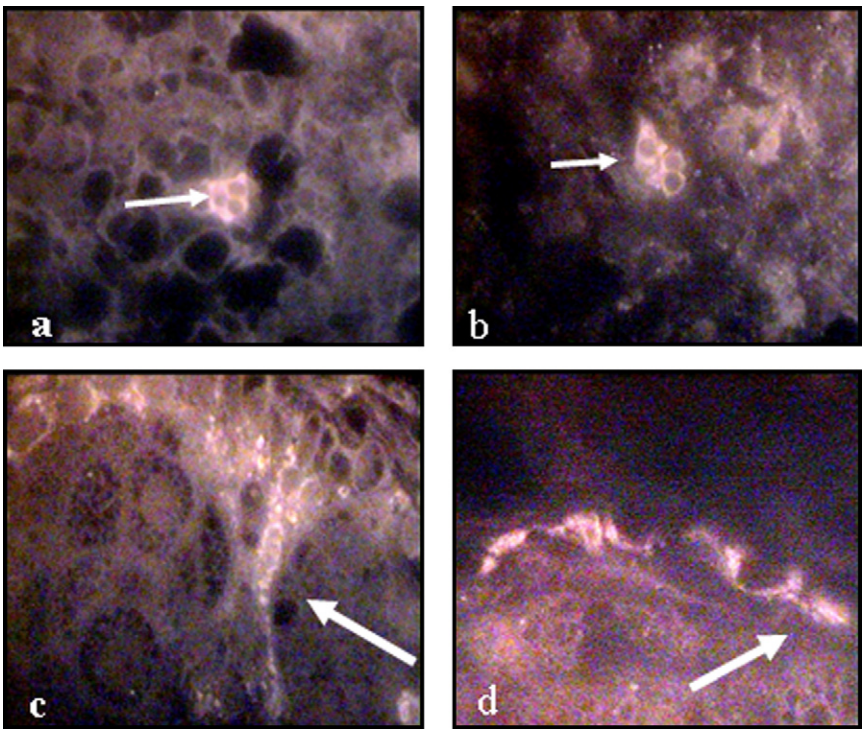

Fig. 4. (a) Skin section of naturally infected sheep showing cells with positive CEV mark, in the scab stage of sebaceous gland, 100×. (b) Skin section of artificially infected goat showing cells with positive CEV mark, in the scab stage of sebaceous gland, 100×. (c) Skin section of naturally infected sheep showing cells with positive CEV mark, in the scab stage of pilose follicle 100×. (d) Skin section of artificially infected goat showing cells with positive CEV mark, in the scab stage of pilose follicle 100×.

infection and recovery. A condition which may be normal in the course of a clinical process which is as rapid as ecthyma and this could be of importance for the negative modulation of the partial immune memory, as much cellular as humoral, and may be a condition related to the cyclical aspect in susceptible flocks.

In the present study, the presence of viral antigens to CEV was confirmed in the progenitor cells of sebaceous glands and in pilose follicles. This agrees in part with previous reports (Jenkinson et al., 1990) where the location of the virus in the skin is mentioned. On the other hand, the quantity of viral antigen dispersed in the epidermis or present in modified keratinocytes of the horny layer was minimal.

In the cells undergoing a process of clear apoptotic characteristic where, inclusion bodies were observed,

Table 2
Number of positive cells for CD4+/CD8+ in artificially infected goat skin with CEV and skin of naturally infected sheep with CEV

Animals	Days post inoculation		
	Day 5[a]	Day 7[a]	Day 9[a]
Epidermis in the inoculation zone data of 3 infected goats	8.3/2.3	16.4/3.6	13.6/2.5
Normal epidermis of 3 infected goats	0	0	0
Epidermis of one normal goat	0	0	0
Epidermis whit lesions of a natural infected sheep	13.3/3.2	17.1/4.2	12.5/2.8
Epidermis of a natural infected sheep whit out lesions	1.3/0.5		

6 fields were counted.
[a] Average/standard deviation.

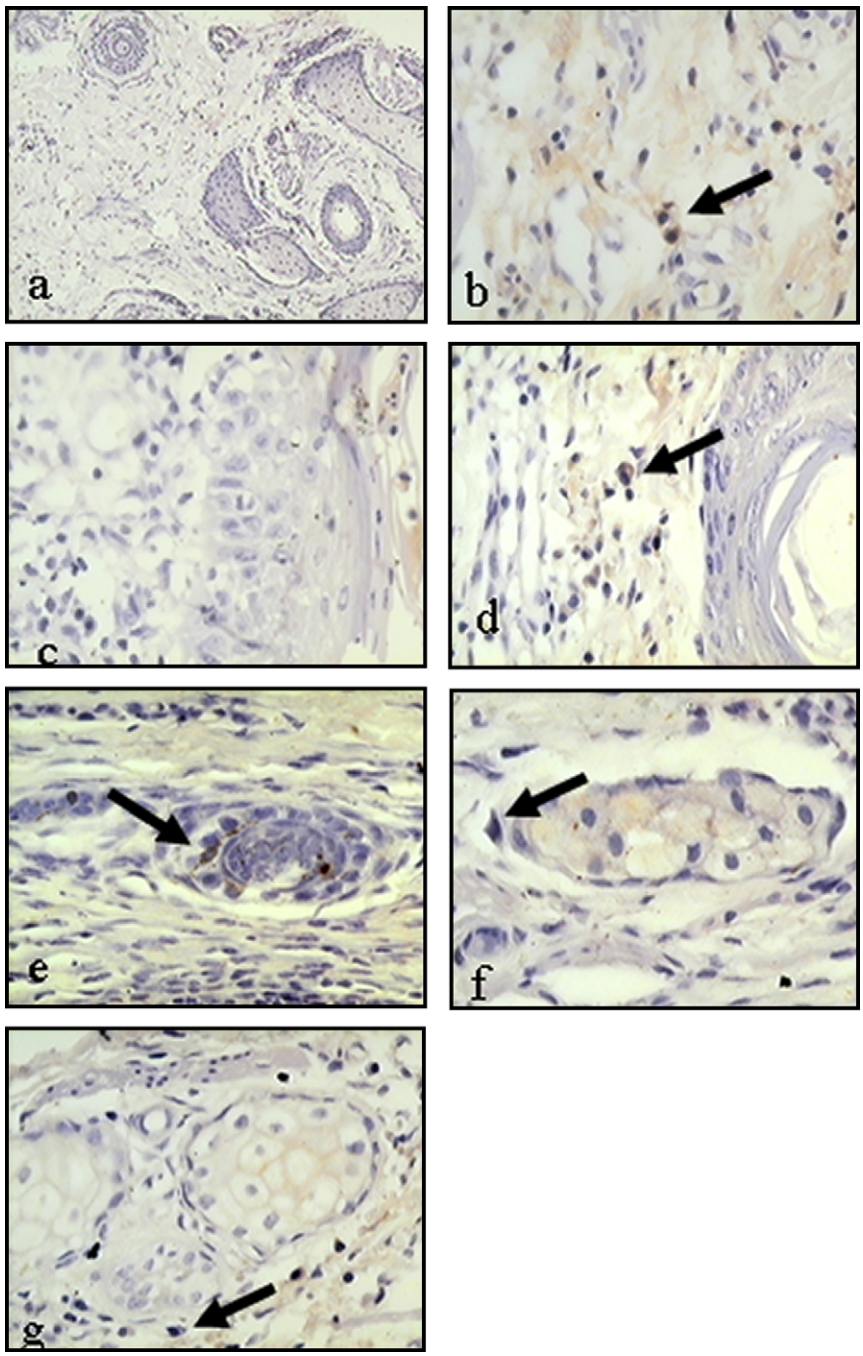

Fig. 5. (a) Skin section of clinically healthy goat. (b) Skin section of artificially infected goat showing CD-4 and CD-8 lymphocytes in hypodermis (arrow), 100×. (c) Skin section of naturally infected sheep showing CD-4 and CD-8 lymphocytes in hypodermis (arrow), 100×. (d) Skin section of naturally infected sheep showing CD-4 and CD-8 lymphocytes in pilose follicle (arrow), 100×. (e) Skin section of artificially infected goat showing CD-4 and CD-8 lymphocytes in pilose follicle (arrow), 100×. (f) Skin section of naturally infected sheep showing CD-4 and CD-8 lymphocytes in relation with the sebaceous glands of the hypodermis (arrow), 100×. (g) Skin section of artificially infected goat showing CD-4 and CD-8 lymphocytes in hypodermis (arrow), 100×.

displaying particles of a similar size to that of the CEV.

In the final stages of the infection process, no relationship was found between the viral antigens and defense cells or cells undergoing apoptosis. This might suggest control on the part of the virus over cells recruited during the humid scab stage, since during healing initiation, both the process of apoptosis and cells of type CD4+ and CD8+ were almost non-existent, and the amount of viral antigen increased in the pilose follicles and sebaceous glands.

The findings reported in this study draw attention to the importance of the apoptotic process. Modulation is evident at least by one or various viral proteins, these acting as signals in order to initiate the process of apoptosis (Kruse and Weber, 2001). The location of viral antigens concentrated in structures related to the common integument and its annexes may permit the permanency of CEV in clinically healthy animals and a possible tolerance towards the chronic presence of immunogenic proteins.

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