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Chicken Anemia Virus Causes Apoptosis of Thymocytes after In Vivo Infection and of Cell Lines after In Vitro Infection

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After infection of 1-day-old chickens, chicken anemia virus (CAV) causes a complete depletion of the thymic cortex by day 14. Since cell death can be caused either by necrosis or by apoptosis, we investigated which type of cell death occurs after in vivo and in vitro infections with CAV. Using electron microscopy and biochemical methods, we demonstrated that CAV induces apoptosis of cortical thymocytes after in vivo infection and of lymphoblastoid cell lines after in vitro infection. At day 13 after in vivo infection, virus-like particles were detected in apoptotic bodies that were absorbed by epithelial cells. These results show that apoptosis, a phenomenon that has been observed for a few other viruses, is also an important phenomenon during the pathogenesis of CAV.

Worldwide, chicken anemia virus (CAV) infection of newly hatched chickens causes considerable health problems and economic losses in the poultry industry. CAV is a small virus with a circular single-stranded DNA genome of 2.3 kb (6, 16, 19). The sequence of the CAV genome is not related to those of any of the virus classes described so far (19). Fourteen days after infection, CAV causes anemia and depletion of the thymus cortex, mainly of the thymocytes (8, 31). Macrophages and stromal and epithelial cells can still be detected, however, with immunohistochemical techniques (13). How the thymus cortex becomes depleted of thymocytes between days 7 and 14 after infection is unknown. Others have described two mechanisms by which nucleated eukaryotic cells die: necrosis and apoptosis (4, 29). Necrosis is considered a pathological reaction that occurs in response to major disturbances in the cellular environment, such as a lytic viral infection (29). Apoptosis, on the other hand, is considered a physiological process that is part of homeostatic regulation during normal tissue turnover (29). However, only a few viruses, such as human immunodeficiency virus (HIV; 2) and bovine herpesvirus 1 (10), have been shown to induce apoptosis.

Necrosis and apoptosis differ from one another in the morphology of the nucleus and in DNA fragmentation. Electron microscopy has shown that when cells die from apoptosis, the chromatin first aggregates next to the nuclear membrane. Then, apoptotic bodies of nuclear material are formed, and these are often absorbed by intact adjacent cells. Furthermore, biochemical analysis has shown that when cells die from apoptosis, the DNA is fragmented into oligonucleosomes (5).

Using electron microscopy and DNA analysis, we investigated whether cell death that occurs after CAV infection of the thymus in vivo and of cell lines in vitro occurs by necrosis or by apoptosis. Our results clearly demonstrate that CAV causes apoptosis of thymocytes after in vivo infection and of two different cell lines after in vitro infection.

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MATERIALS AND METHODS

Viruses and cell lines. Two chicken lymphoblastoid cell lines were used: the T-cell line MDCC-MSB1 transformed by Marek's disease virus (1) and the B-cell line 1104-X5 induced by avian leukemia virus (11). These cells were infected with a multiplicity of infection of 0.1 to 1 50% tissue culture infective doses of CAV and harvested 48 h after infection. The cells were infected with isolate CAV-Cux-1, originally recovered from a flock in Germany (27, 28); with isolates CAV-T-1704 and CAV-Del-S, originally from the United States; or with several Dutch isolates (20).

Animals. Specific-pathogen-free White Leghorn chickens were infected with 10^6 50% tissue culture infective doses of CAV-Cux-1 at day 1 of age. At days 6, 10, 13, and 20, thymuses of CAV-infected chickens and control chickens were removed.

Electron microscopy. Uninfected and CAV-Cux-1-infected MDCC-MSB1 and 1104-X5 cells were collected, washed in medium, and pelleted. Thymuses were cut in tissue blocks of ± 1 mm^3. Cell pellets and thymus blocks were fixed in a mixture of 0.8% glutaraldehyde and 0.8% osmium tetroxide in a veronal acetate buffer, washed, and then stained in 1% uranyl acetate. Further processing for electron microscopy was as described previously, with microwave stimulation (22). Ultrathin sections, contrasted with uranyl acetate and lead citrate, were examined in a Philips CM10 transmission electron microscope.

Analysis of total DNA. DNA was extracted from mock- and CAV-infected MDCC-MSB1 and 1104-X5 cells and from thymuses of CAV-infected and specific-pathogen-free chickens. Samples were suspended in 20 mM Tris-HCl (pH 7.5) containing 2 mM EDTA, 0.2% sodium dodecyl sulfate, and 0.6 mg of proteinase K per ml. The suspensions were incubated for 1 h at 37°C and subsequently extracted once with phenol and once with phenol-chloroform-isoamylalcohol (25:24:1). The DNA was precipitated with ethanol and suspended in 10 mM Tris-HCl (pH 7.5)–1 mM EDTA. Aliquots of the DNA were fractionated on a 1.5% agarose–ethidium bromide gel.

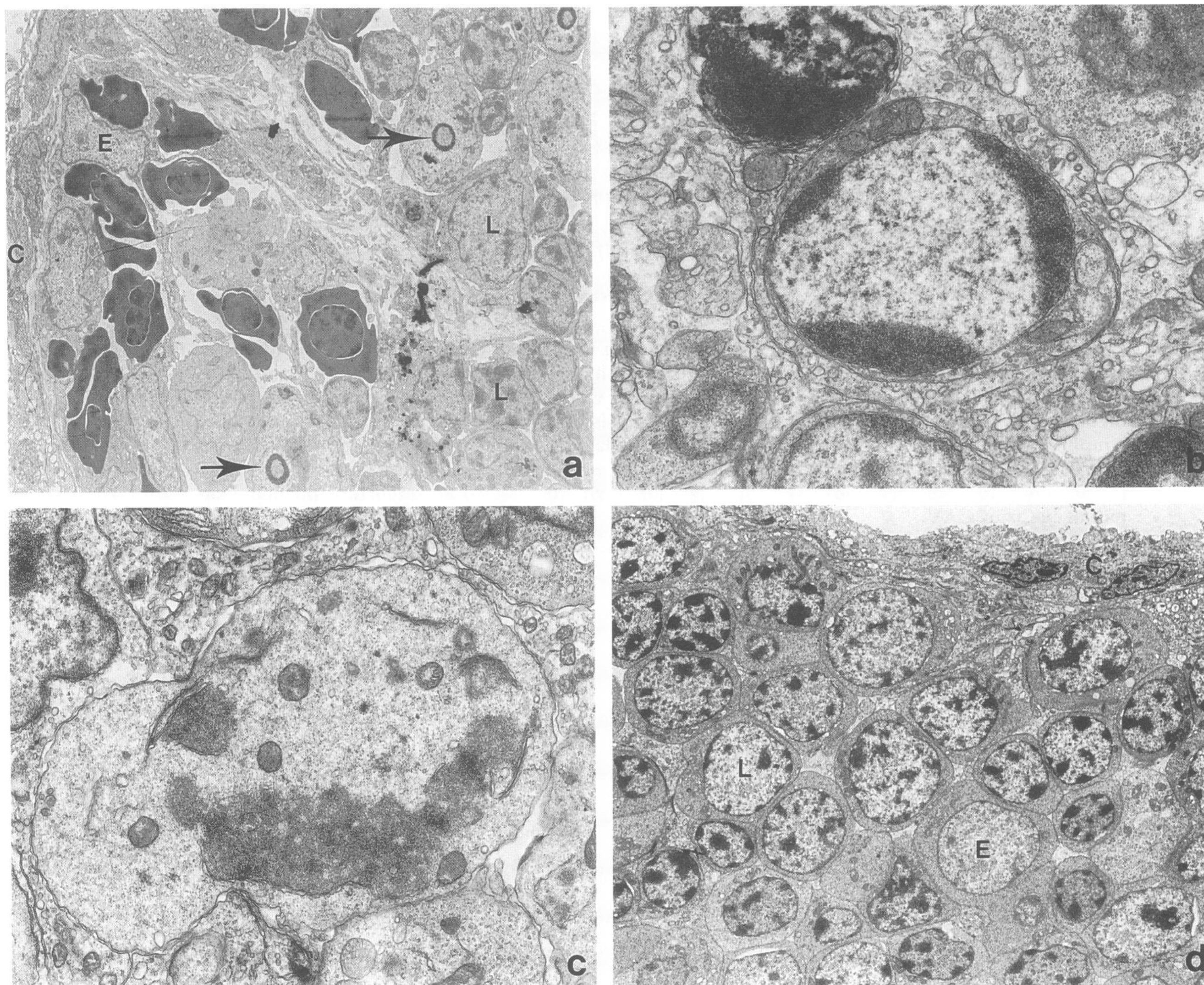


FIG. 1. Electron microscopical examination of the thymus at day 6 after in vivo infection with CAV (a through c) and of control thymus (d). (a) In the cortex, some thymocytes contain electron-dense rings in their nuclei (arrows). C, thymic capsule; E, epithelial cell; L, lymphocyte. Magnification, ×2,400. (b) Apoptotic thymocyte with its chromatin aggregated in large, dark, condensed masses that abut the nuclear membrane. Magnification, ×13,000. (c) Thymocyte with its nucleus broken up into a number of discrete fragments partly surrounded by double nuclear membranes. Magnification, ×9,900. (d) In the cortexes of uninfected chickens, lymphocytes (L) and epithelial cells (E) are densely packed together. C, thymic capsule. Magnification, ×2,400.

RESULTS

Electron microscopy of thymuses after in vivo infection. At day 6 after infection, many thymocytes with electron-dense rings in their nuclei were detected at the outer border of the cortex (Fig. 1a). These rings were not observed in control chickens and have been attributed by others to CAV infection (7, 17). We detected some thymocytes scattered through the outer cortex whose chromatin was aggregated in large, dark, compact masses that abutted the nuclear membrane (Fig. 1b). In addition, the nuclei of some thymocytes were broken into a number of discrete fragments partly surrounded by double nuclear membranes (Fig. 1c).

At day 10 after infection, only a few thymocytes with electron-dense rings in their nuclei were located in the deeper cortex. Thymocytes with apoptotic nuclei (as shown in Fig. 1b) were detected throughout the entire cortex. In addition, clusters of dead cells were detected (Fig. 2a). More or less round apoptotic bodies were detected sporadically in the cytoplasms of epithelial cells (Fig. 2b). The phenomena of chromatin aggregation, nuclear fragmentation, and apoptotic bodies were not observed in thymuses of age-matched control chickens (data not shown).

At day 13 after infection, the cortex was severely depleted of thymocytes, whereas nonlymphoid cells, such as epithelial cells, were still present. Thymocytes with electron-dense rings in their nuclei were no longer detected. In addition, only a few apoptotic bodies were detected. In contrast, we detected many large cells with lucent cytoplasms; the cells contained many small dark granules separated from the cytoplasms by double membranes (Fig. 3a). Because these large lucent cells had tonofilaments and desmosomes, they were considered epithelial cells. In some of these granules,

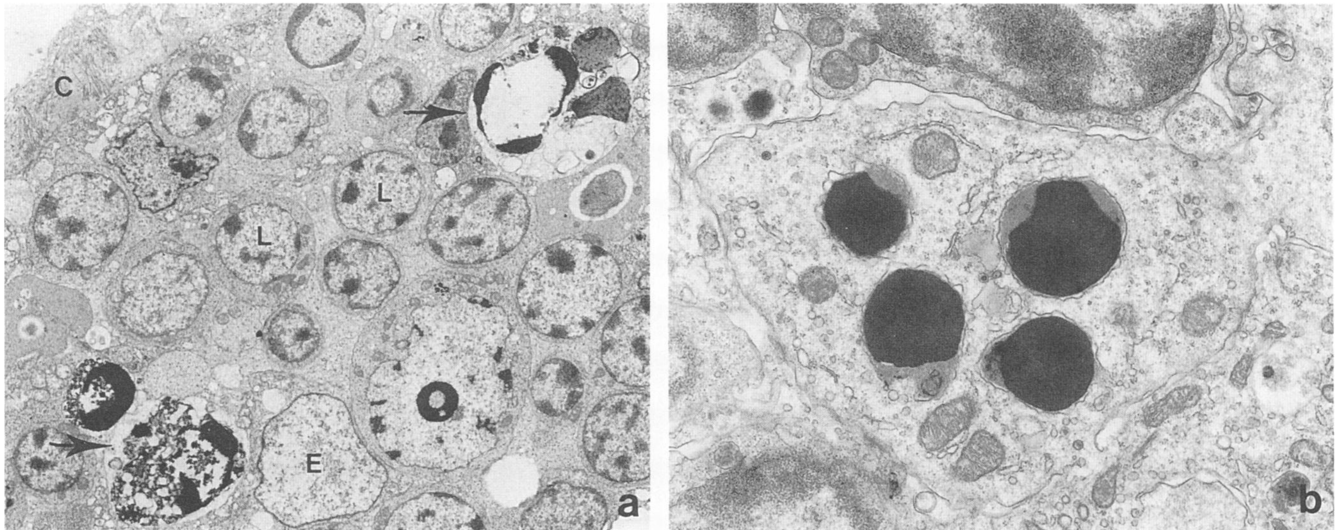


FIG. 2. Electron microscopical examination of the thymus at day 10 after in vivo infection with CAV. (a) Apart from thymocytes with electron-dense rings and apoptotic nuclei, clusters of dead cells were detected (arrows). C, thymic capsule; E, epithelial cell; L, lymphocyte. Magnification, ×3,300. (b) More or less round apoptotic bodies in vacuoles in cytoplasm. Magnification, ×13,000.

clusters of virus-like particles were detected (Fig. 3b). The virus-like particles consisted of dark stained cores surrounded by thin unilamellar membranes. They were between 35 and 40 nm in diameter. These particles were not detected earlier in the infections or in control chickens.

At day 20 after infection, the cortex seemed comparable to those of control chickens in that many thymocytes were present. At various sites, however, epithelial cells still contained many dark granules, which are the remnants of the CAV infection (Fig. 4).

Electron microscopy of cell lines after in vitro infection. To exclude the possibility that apoptosis is induced by CAV only during in vivo infections, we also examined the type of cell death that occurs after in vitro CAV infection of lymphoblastoid cell lines. Like the thymus at day 6 after CAV infection, both the B-cell line (1104-X5) and the T-cell line (MDCC-MSB1) contained many cells with characteristic electron-dense rings in their nuclei at 48 h after infection (Fig. 5a). In addition, some cells showed apoptosis. These cells were characterized by the presence of multiple nuclei with chromatin condensation at one side of the nucleus (Fig. 5b).

DNA analysis of thymuses and cell lines. The cell death caused by CAV was investigated further by agarose gel electrophoresis of total DNA (Fig. 6). DNA from CAV-infected MDCC-MSB1 cells showed the apoptosis-specific pattern of nucleosomal laddering. This laddering was absent in DNA from uninfected MDCC-MSB1 cells. DNA isolated from MDCC-MSB1 cells which had died from being cultured at far too high densities or at a nonphysiological pH provided

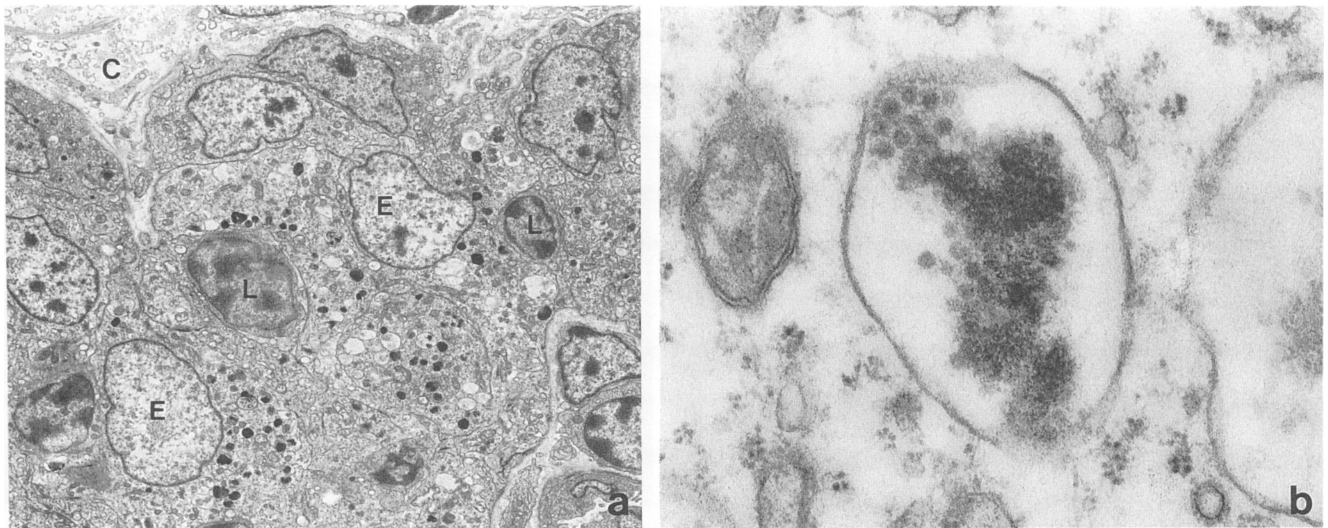


FIG. 3. Electron microscopical examination of the thymus at day 13 after in vivo infection with CAV. (a) The cortex is severely depleted of lymphocytes (L). Epithelial cells (E) contain small dark granules in their cytoplasms. C, thymic capsule. Magnification, ×3,300. (b) Clusters of virus-like particles 35 to 40 nm in diameter in a vacuole. Magnification, ×58,000.

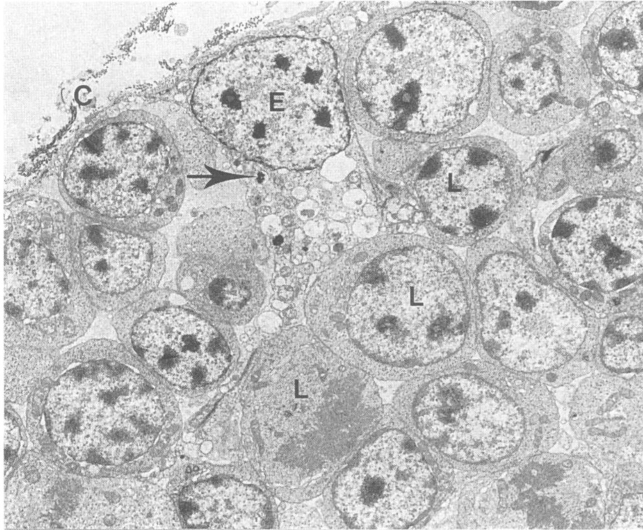


FIG. 4. Electron microscopical examination of the thymus at day 20 after in vivo infection with CAV. Many lymphocytes (L), often in mitosis, are visible in the cortex. An epithelial cell (E) contains some dark granules of cell debris (arrow). C, thymic capsule. Magnification, ×3,300.

a smear typical for necrosis (data not shown). Although various strains of CAV were used (CAV-Cux-1 [Fig. 6, lane 2], CAV-T-1704 and CAV-Del-S [lane 3], and Dutch isolates [lanes 4 and 5]), nucleosomal laddering was detected in all lanes. After infection of the B-cell line 1104-X5, CAV was shown to cause nucleosomal laddering in a non-T-cell line as well (lanes 6 and 7). After in vivo infection with various field strains of CAV, thymic DNAs from these chickens showed nucleosomal laddering (lanes 9 through 13). Laddering was not detected in thymic DNAs of uninfected chickens (lane 8).

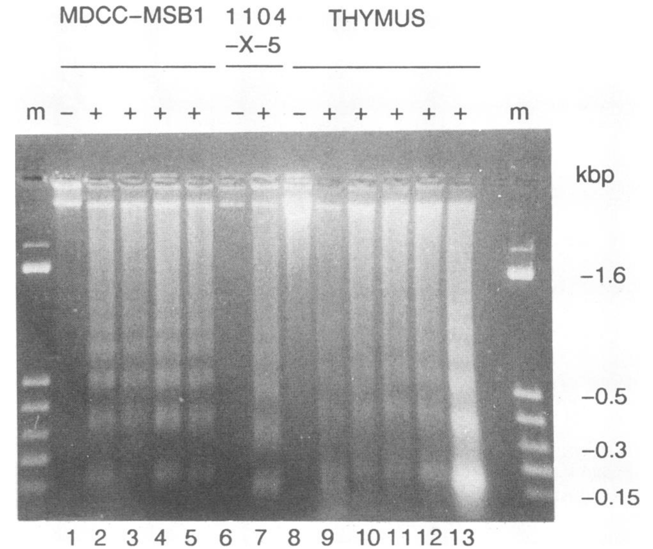
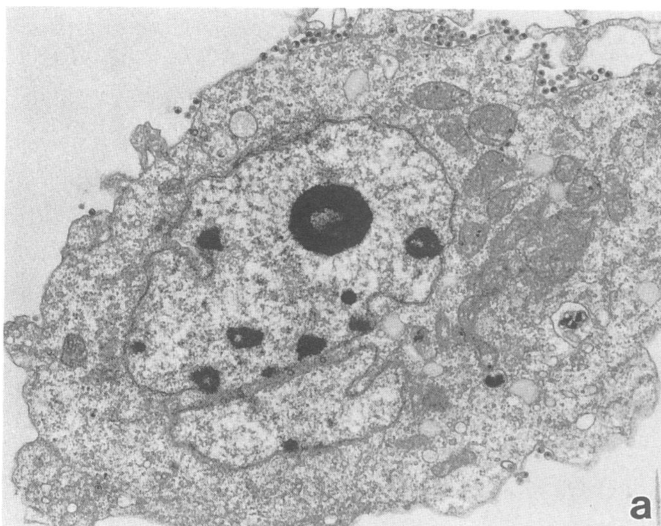


FIG. 6. DNA fragmentation in thymus and cell lines undergoing CAV-mediated cell death. DNAs from MDCC-MSB1 cells, 1104-X5 cells, or thymuses from chickens were analyzed on a 1.5% agarose gel. MDCC-MSB1 cells were mock infected (lane 1) or infected with isolate CAV-Cux-1 (lane 2), CAV-T-1704 and CAV-Del-S (lane 3), or Dutch CAV (lanes 4 and 5). 1104-X5 cells were mock infected (lane 6) or infected with CAV-Cux-1 (lane 7). Cells were infected and harvested as described in Materials and Methods. Thymuses were collected from control chickens (lane 8) or from chickens from various flocks in The Netherlands that had clinical signs of CAV at an age of 2 to 3 weeks (lanes 9 through 13). −, mock infected; +, CAV infected; m, DNA markers (pAT153-*Hin*fI fragments [14]).

DISCUSSION

Using electron microscopy and DNA analysis, we investigated whether cell death after CAV infection of the thymus in vivo and of cell lines in vitro occurs by necrosis or by



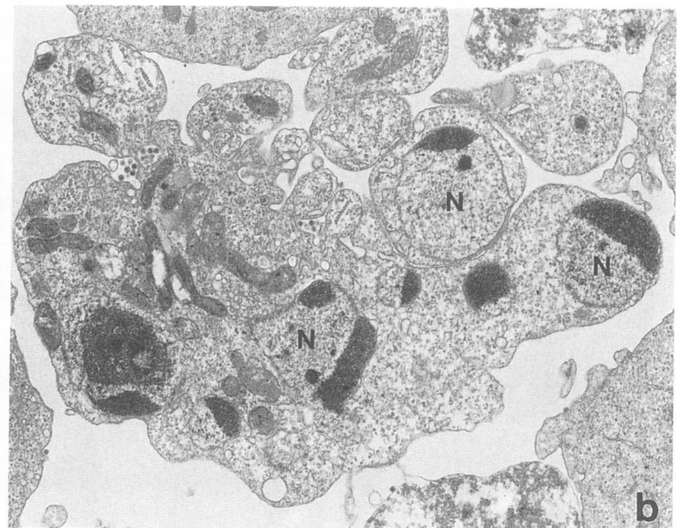


FIG. 5. Electron microscopical examination of cell lines after in vitro infection with CAV. (a) 1104-X5 cell with characteristic electron-dense rings in its nucleus. Avian leukosis virus, the transforming virus of this cell line, is present outside of the cell. Magnification, ×9,900. (b) Apoptotic cell of the 1104-X5 cell line with multiple nuclei (N) and chromatin condensations at the sides of the nuclei. Magnification, ×7,300.

apoptosis. Our results clearly demonstrate that CAV causes apoptosis of thymocytes after in vivo infection and of two different cell lines after in vitro infection. DNA analysis was performed on cells that were infected with CAV isolates recovered in Germany, the United States, and The Netherlands. All CAV isolates induced identical patterns of apoptosis in infected cells. These results indicate that apoptosis might be a general phenomenon of CAV infections worldwide. Apoptosis is a process leading to cell death that causes no damage to surrounding cells and occurs rapidly (4, 29). Indeed, the total cortex becomes depleted between days 7 and 14 after infection (8, 13).

The question arises as to how CAV induces apoptosis. Apoptosis may represent a concomitant process during infection. On the other hand, the induction of apoptosis may also represent an obligatory phase of CAV infection. The hypothesis of concomitance is strengthened by the fact that apoptosis can also be caused by several nonviral stimuli, such as lymphotoxin, tumor necrosis factor, and glucocorticoids, and also by a lack of growth factors. These external stimuli can converge on a common intracellular pathway, resulting in the activation of endogenous endonuclease (15). In general during infections, the physiological levels of lymphotoxin, glucocorticoids, and growth factors become disturbed. Moreover, apoptosis can be activated via receptors on the cell membrane. Receptor-mediated activation of apoptosis has been shown for T cells (26) and thymocytes (23). In addition, antibodies that bind to the cell surface APO-1 or Fas proteins may mediate apoptosis (12, 25). Furthermore, in HIV-infected patients, the interaction of soluble HIV gp120 envelope protein with CD4 molecules in serum and lymph has been proposed as causing a defective signal transduction that leads to apoptosis in several cell populations, including the T-helper population (21). Analogous to HIV proteins, a CAV protein might induce apoptosis by binding to a specific receptor on thymocytes. The CAV genome contains three open reading frames that encode three different proteins (19). We have strong evidence that these open reading frames are expressed in CAV-infected cells, for in vitro expression of them (data not shown) yielded CAV protein products similar to those observed in CAV-infected cells (3). The 50-kDa protein is most likely the capsid protein, whereas the functions of the 30- and 16-kDa proteins are unknown. One of these CAV proteins might induce apoptosis by binding to a surface molecule on thymocytes.

The second hypothesis, i.e., that apoptosis is an obligatory phase of the infection, can also be supported. First, CAV is strongly specific for bone marrow, thymus, and bursa tissue, and it can also be cultured on only a few cell lines (30). In contrast to many leukocytes, such as mature T cells, thymocytes are depleted by apoptosis during their normal in vivo development. Most cell lines used to culture CAV are lymphoblastoid cell lines of thymic or bursal origin. In the bursa, many cells are also depleted by apoptosis during their normal development (18). Since the bone marrow is also susceptible to CAV infection, it is important to know whether apoptosis occurs in the bone marrow during normal hemopoiesis. As far as we know, this question has not been investigated. A publication by Goryo and coworkers (9), however, describes a phagocytosed erythrocyte with "its nuclear chromatin condensed in the peripheral region of the nuclear membrane" that was found in the bone marrow at day 6 after CAV infection. Although those authors do not refer to this phenomenon as apoptosis, the morphology of the nucleus is similar to that described in this study. Thus, it appears that the ability of CAV to replicate in a certain cell type coincides with the ability of this cell type to undergo apoptosis. Apoptosis may also be an obligatory phenomenon during CAV infection, because virus-like particles are detected only in degraded apoptotic bodies, which are absorbed by epithelial cells. Although the degraded apoptotic bodies have been observed by investigators, no mention has been made of any virus-like particles (7). Instead, Goryo and coworkers described virus-like particles 14 nm in diameter in the nucleus at day 7 after infection (7). After purification over a CsCl gradient, the CAV particles, however, are generally accepted to be between 23 and 27 nm in diameter (6, 17, 24). The virus-like particles detected in thymic epithelial cells in this study seem to consist of cores with unilamellar membranes around them. These virus-like particles are larger than those found after CsCl gradient purification. The difference may be caused by the unilamellar membrane. It seems clear, however, that this membrane is lost during the purification of CAV, which involves chloroform treatment and CsCl gradient centrifugation. The loss of this membrane does not impair the infectivity of CAV. The size of the core, although difficult to determine, seems commensurate with that of virus particles measured after CsCl gradient purification. We therefore think that the virus-like particles seen in epithelial cells at day 14 after infection are newly formed CAV particles.

We conclude that CAV causes apoptosis in thymocytes after in vivo infection and in cell lines after in vitro infection. Whether apoptosis is an essential part of the replication of CAV or is merely a concomitant effect should be investigated further.

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