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NOTES

Apoptosis and T-Cell Depletion during Feline Infectious Peritonitis

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Cats that have succumbed to feline infectious peritonitis, an immune-mediated disease caused by variants of feline coronaviruses, show apoptosis and T-cell depletion in their lymphoid organs. The ascitic fluid that develops in the course of the condition causes apoptosis in vitro but only in activated T cells. Since feline infectious peritonitis virus does not infect T cells, and viral proteins did not inhibit T-cell proliferation, we postulate that soluble mediators released during the infection cause apoptosis and T-cell depletion.

Feline infectious peritonitis (FIP) is a sporadic, mostly fatal disease in domestic cats and exotic felids; it is characterized by fibrinous serositis with accumulation of fluids in body cavities, disseminated pyogranuloma formation, hypergammaglobulinemia, and formation of immune complexes (for a review, see reference 3). It is the consequence of an infection with feline coronavirus variants that can replicate in macrophages (23, 32); viral antigen can be detected in macrophages of pyogranulomatous lesions in diverse organs including the liver, spleen, and kidneys (23). During the late stages of FIP, antibody titers may rise to high levels, circulating immune complexes appear, and complement is activated (13). These immune complexes and infection-enhancing antibodies directed against the viral spike protein (leading to "early death") have been postulated to play a role in the immunopathogenesis of FIP (3, 36). T-cell-mediated immunity, on the other hand, is considered essential in protection (23).

Experimental inoculation with the highly virulent FIP virus (FIPV) strain 79-1146 kills cats between 20 and 30 days postinfection (p.i.) (36). We collected organ samples from cats (oronasal inoculation, 1,000 PFU, strain 79-1146 [$n = 4$] or feline enteric coronavirus strain 79-1683 [$n = 4$]; control cats [$n = 2$]) at 22 days p.i., fixed them in 4% formaldehyde, and embedded them in paraffin; at this time point the FIPV-infected cats exhibited clinical symptoms. We have shown before that FIPV RNAs can be detected in the plasma of infected cats from day 4 p.i. onwards using reverse transcriptase PCR (11). When examining the spleen and mesenterial lymph nodes of FIP cats we observed opaquely stained nuclei, indicative of chromatin condensation, a morphological hallmark of apoptosis (Fig. 1A). Apoptosis, or programmed cell death, is a suicide mechanism that constitutes the physiological response of cells to damaging stimuli (2). It involves the activation of an endogenous endonuclease, resulting in the fragmentation of cellular DNA. Fragmented DNA was detected by using the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-biotin nick end-labelling (TUNEL) method (6, 34). Deparaffinated tissue sections were washed with TdT buffer (0.5 M cacodylate [pH 6.8], 1 mM $CoCl_2$, 0.5 mM dithiothreitol, 0.05% bovine serum albumin [BSA], 0.15 M NaCl), incubated with 4 μM biotin-conjugated dUTP (Boehringer Mannheim) and 10 U of TdT (Promega) in 50 μl of TdT buffer (1 h, 37°C), and incubated with extravidine peroxidase or goat anti-biotin-phosphatase (kindly provided by P. van der Meide, Rijswijk, The Netherlands). Tissue sections were also examined by indirect immunoperoxidase staining for viral antigen by using a monoclonal antibody specific for the FIPV nucleocapsid protein (COR 12) or for T-cell markers by using rabbit anti-CD3 antibodies (Dako), or they were incubated with anti-cat peroxidase-labelled immunoglobulin to localize B cells. Selected tissue sections were used for double-labelling studies. Bound peroxidase and phosphatase was detected with diamino-benzidine, amino-ethyl-carbazole, and fast Blue BB salt (Sigma), and slides were counterstained with hematoxylin.

Using the TUNEL method we confirmed that large numbers of lymphocytes were indeed apoptotic (Fig. 1B and D through F) while only a few were seen in the noninfected control animals (Fig. 1C). The spleen and mesenterial lymph nodes were much more affected than nondraining (e.g., mandibular) lymph nodes. No labelling was observed when the TdT enzyme was omitted (Fig. 1A). The apoptotic signal was detected either in the nucleus (Fig. 1E and F) or, especially in the lymph nodes, in the cytoplasm of macrophages (Fig. 1D), since many cells undergoing programmed cell death are removed by phagocytosis before the process culminates in the apoptotic morphology of the nucleus (2). DNA fragmentation was observed both in T- and B-cell areas of lymph nodes and spleen including germinal centers, red pulp, cortex/paracortex, and sinuses, but also in the omentum, the pyogranulomas, and the thymus. Similar results were obtained with field cases of FIP (not shown).

Apoptosis may result directly from infection, as described for ortho- and paramyxoviruses and alpha-, retro-, and circoviruses in vitro (5, 12, 14–16, 35). This is not the case in FIP, where double staining of sections showed apoptotic nuclei and FIPV-antigen-positive cytoplasm in different cells (Fig. 1F). Moreover, infection of cultured *Felis cattus* whole fetus (fcwf) cells also did not result in apoptosis (9). This would be in line

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with observations made by Shibata et al. (31) showing that mouse hepatitis virus (MHV), another coronavirus, does not induce apoptosis upon infection of cells in vitro; MHV-infected cells undergo apoptosis only after recognition by specific T cells. The presence of FIPV-antigen-positive cells which do not display apoptosis suggests that they are not killed by T cells. Thus, the depletion of T cells during FIP may impede the removal of infected cells through apoptosis. In contrast, during MHV infection viral-antigen-positive cells which are apoptotic can be detected in vivo (9). Apoptosis in cats succumbing to FIP may be caused through a bystander mechanism; the apoptotic cells are most likely lymphocytes, whereas only macrophages and intestinal epithelial cells are found to be infected (23).

The phenotype of the apoptotic cells was difficult to determine; they only occasionally stained for CD3 or immunoglobulin. Membrane changes during the apoptotic process and tissue processing (fixation and paraffin embedding) probably diminish preservation of surface epitopes (22). However, a marked depletion of $CD3^+$ lymphocytes and involution of germinal centers were noticed (Fig. 2B and D) which were more pronounced in the advanced stages of FIP. In contrast, cats infected with a feline enteric coronavirus strain of low virulence (79-1683) were able to control the infection and exhibited enlarged germinal centers (Fig. 2C). We also did not find any evidence for T-cell redistribution in FIP cats; the granulomatous lesions and the ascitic fluid contained only a few $CD3^+$ cells, but large numbers of plasma cells were found in the omentum and ascitic fluid. Hypergammaglobulinemia and immune complex formation are observed in the late stages of FIP (13), and apoptosis of B cells is therefore unlikely to affect humoral immune responses. Although apoptosis is a physiological process and not specific for a pathologic condition (19, 22), in FIP it may result in a dysregulation of lymphocyte turnover followed by T-cell deficiency, which may explain the observed lymphopenia and thymus atrophy (23). Also during MHV infection involution of the thymus through apoptosis has been demonstrated (7). Moreover, at 18 days p.i. peripheral blood lymphocyte (PBL) counts in experimentally infected cats were very low ($0.37 \times 10^6 \pm 0.25 \times 10^6$ cells per ml) compared with control cats ($2.25 \times 10^6 \pm 0.35 \times 10^6$ cells per ml).

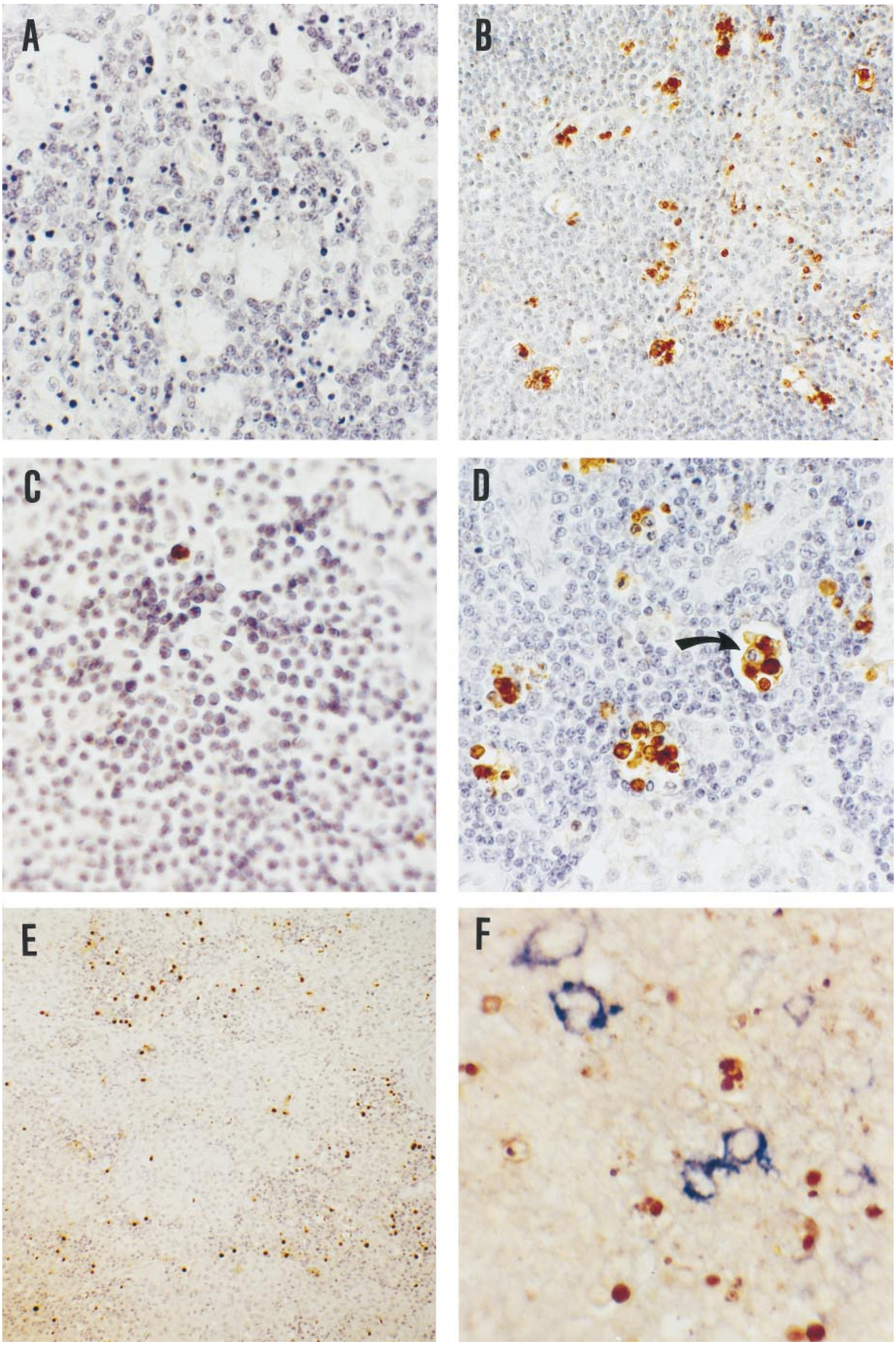
To determine whether T cells from FIP cats are susceptible to activation-induced apoptosis, we analyzed their response to mitogen stimulation in vitro. PBL and single-cell suspensions from lymph nodes and spleens of cats euthanized in extremis (confirmed FIP cases by postmortem examination) were prepared. For the TUNEL test, cells cultured in 24-well clusters (10^6 cells per ml) were fixed after 24 h in 2% formaldehyde (10 min at room temperature) followed by 70% ethanol (10 min at room temperature). After being washed with TdT buffer (0.5 M cacodylate [pH 6.8], 1 mM $CoCl_2$, 0.5 mM dithiothreitol, 0.05% BSA, 0.15 M NaCl), the cells were incubated with 4 μM biotin-conjugated dUTP (Boehringer Mannheim) and 10 U of TdT (Promega) in 50 μl of TdT buffer (1 h, 37°C), incubated with avidin-fluorescein isothiocyanate (Sigma) in 0.1% Triton X-100, and examined in a fluorescence microscope. For stimulation experiments, cells were cultured in 96-well round-bottom plates at a density of 10^5 per well in RPMI 1640 medium containing 10% fetal calf serum, 2 mM L-glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml; concanavalin A (ConA) (5 μg/ml) was added, and after a 48-h incubation at 37°C in a 5% CO_2 atmosphere the cultures were labelled with 1.0 μCi of [^{3}H]thymidine (Amersham, Den Bosch, The Netherlands) per ml for another 16 h. Cells were harvested on glass filters, and the incorporated radioactivity was measured in a Betaplate scintillation counter (LKB-Wallac). The data represent the mean values ± standard errors of the mean of [^{3}H]thymidine uptake of triplicate cultures.

As shown in Fig. 3A, a high percentage of mesenterial lymph node lymphocytes isolated from FIP cats and stimulated with ConA in vitro were killed through apoptosis, indicating that they had been programmed for death following their activation. In contrast, ConA-stimulated lymphocytes from the mandibular lymph nodes contained low percentages of apoptotic cells. Using Hoechst 33342 (Sigma), which detects early apoptotic cells (4), we could confirm that the cells displayed apoptosis (9). Also in infections with, e.g., retro- and arenaviruses, apoptosis of T cells has been demonstrated upon stimulation of the T-cell receptor/CD3 complex (18, 26, 29). Consistent with earlier reports, which demonstrated that ConA- and FIPV-specific PBL responses are inhibited in FIP cats (33), we observed suppressed blastogenic responses to ConA of FIP mesenterial lymph node cells (Fig. 3B). Similar results were obtained using spleen cells and PBL (not shown), whereas T cells derived from the mandibular lymph nodes did respond to mitogenic stimulation (Fig. 3B). T-cell depletion may have influenced the observed unresponsiveness of lymphocytes to ConA. Nevertheless, mesenterial lymph node T cells from diseased cats were activated since they produced interleukin-2 (IL-2) (not shown).

Several mechanisms including inappropriate cell signalling, specific functions of viral proteins, and the release of corticosteroids and cytokines may account for the observed apoptosis in vivo (1, 2, 8, 21, 37–39). Viral antigen, when displayed at high concentrations by antigen-presenting cells, may cause T-cell depletion (21), which may contribute to the lymphocyte apoptosis observed in FIP. However, only small numbers of infected macrophages are present, mainly in pyogranulomatous lesions and rarely in the lymph nodes, in FIP cases. Furthermore, apoptosis seemed to be nonspecific, since virtually all T cells were depleted from the spleen and draining lymph nodes. We therefore tested an alternative explanation: are soluble mediators able to suppress T-cell responses through apoptosis? In order to analyze the effect of different stimuli on T cells, we used feline thymocytes which were kept in culture with ConA (5 μg/ml) and 50 to 100 U of recombinant human IL-2 per ml. Before use, cells were extensively washed to remove dead cells, cultured in 96-well flat-bottom plates (Nunc) at a density of 10^6/ml in the presence of IL-2 (20 U/ml), and treated with sera or ascitic fluid (inactivated at 56°C for 30 min) at a 1:20 dilution. Conditioned medium from fcwf cells infected with FIPV strain 79-1146, from splenocytes, and from cells which were sedimented from FIP ascitic fluid were collected after 48 h and similarly tested for suppressive activity. After 24 h cells were labelled with 1 μCi of [^{3}H]thymidine per ml for 16 h or processed for detection of DNA fragmentation by using the TUNEL method.

Heat-inactivated ascitic fluid from FIP cats was found to

FIG. 1. Apoptosis in lymph nodes (A through D) and spleen (E and F) of FIPV (A, B, and D through F)- and mock-infected cats (C). Mesenterial lymph nodes and spleens from FIPV-infected cats were analyzed for apoptotic cells by using the TUNEL method in the absence (A) or presence of TdT (B through F). The arrow in panel D indicates apoptotic cells phagocytized by a macrophage. Spleen tissue from an FIPV-infected cat was double stained for FIPV antigen (blue) and apoptotic cells (brown, panel F). Magnifications, ×34 (panel E), ×69 (panel B), and ×138 (panels A, C, D, and F).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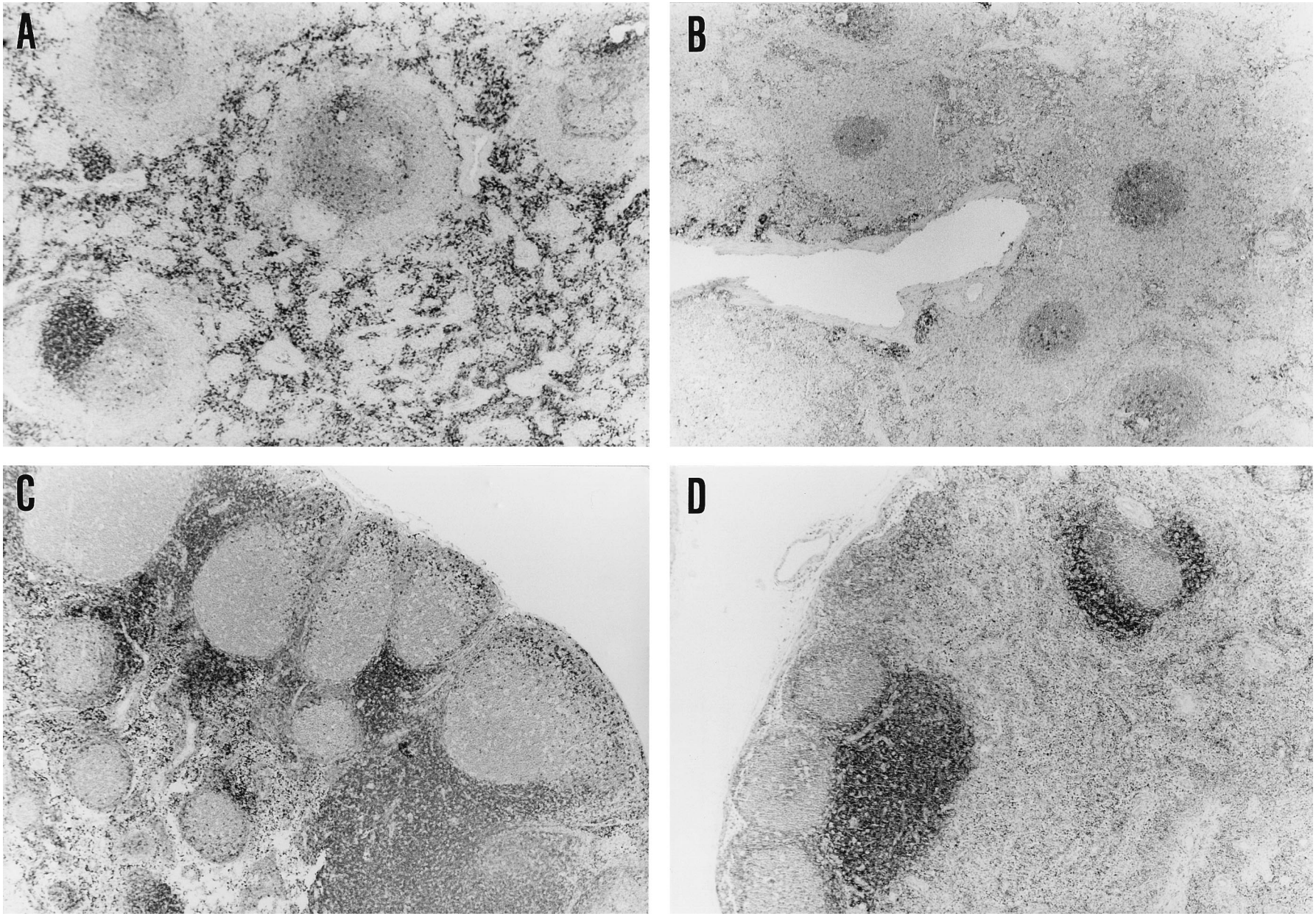

FIG. 2. Depletion of lymphocytes in the spleen and lymph nodes of FIPV-infected cats. Spleens (A and B) and lymph nodes (C and D) from control cats (A), FIPV-infected cats (B, D) and feline enteric coronavirus-infected cats (C) were analyzed for the presence of $CD3^+$ cells. Magnification, ×25.

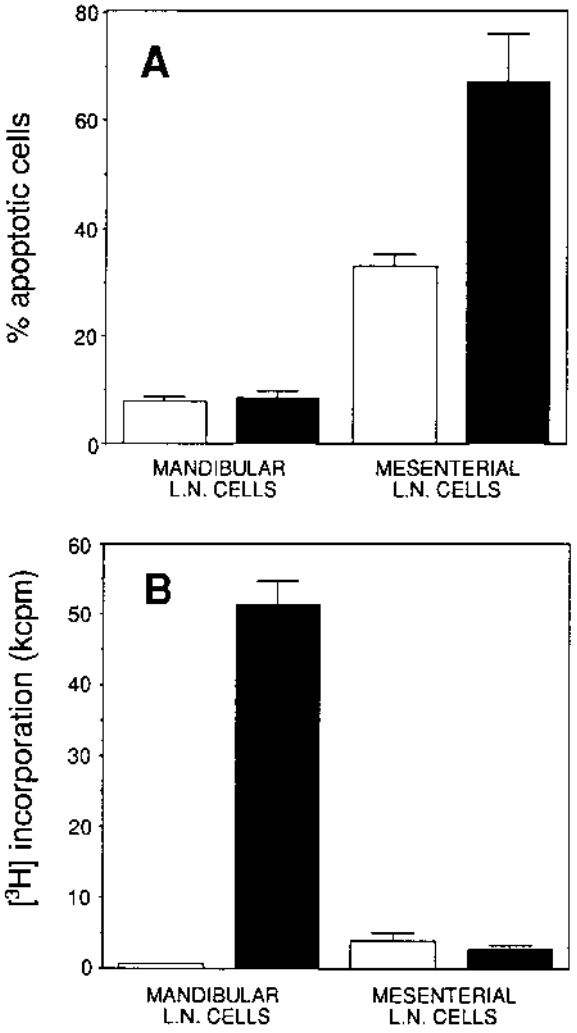

FIG. 3. Response of lymph node cells from FIPV-infected cats to mitogen activation. Percentages of apoptotic cells (A) and proliferative responses (B) of mandibular and mesenterial lymph node cells from an FIPV-infected cat to medium without (open bar) and with ConA (solid bar) are shown.

suppress T-cell proliferation (Fig. 4), as did sera from infected cats (not shown). Since supernatant from FIPV-infected cell cultures did not inhibit feline T-cell proliferation, a direct effect of virions or viral proteins can be excluded. The soluble mediators are very likely released by leukocytes since conditioned medium from cells recovered from the ascitic fluid (CM-ASC) inhibited T-cell proliferation (Fig. 4). Conditioned medium from splenocytes also inhibited lymphocyte proliferation (20,505 ± 1,021 cpm compared with 48,114 ± 2,935 cpm in control cultures). In contrast, conditioned medium from splenocyte cultures of uninfected cats did not significantly inhibit lymphocyte proliferation (45,317 ± 2,217 cpm).

Next, we tested whether soluble mediators suppressed T-cell responses through apoptosis. ConA blasts derived from PBL from a specific-pathogen-free cat and kept in culture for several months in the presence of ConA–IL-2 displayed genomic DNA degradation after treatment with CM-ASC, and their proliferation was inhibited (Fig. 5). ConA was used as a positive control. CM-ASC is not cytotoxic as such; when unstimulated T cells were used no apoptosis was observed (Fig. 5). Ascitic fluid exerted similar effects and did not affect cell viability of primary cat macrophages or fcwf cells (not shown). These data suggest that activated but not resting lymphocytes are killed by factors in the ascitic fluid.

Several viruses cause apoptosis of T cells (14, 15, 35). Here we show that a virus which does not replicate in T cells can nevertheless induce T-cell depletion. T-cell suppression may enhance viral replication during FIP. Several observations support the hypothesis that cell-mediated immunity plays a decisive role in the protection against FIP. Firstly, the clinical incidence of FIP is increased by concurrent infection with feline leukemia virus or feline immunodeficiency virus (25, 28), which both suppress cell-mediated immunity. Secondly, blastogenic responses and delayed hypersensitivity responses to FIPV can be detected in FIP-recovered cats (24). Thirdly, there is a more severe disease in thymectomized versus control kittens infected with FIPV (10). Depletion of T cells through indirect mechanisms rather than through replication also has been postulated to play an important role during human immunodeficiency virus infection (20); human immunodeficiency virus proteins are thought to cause apoptosis of $CD4^+$ cells (1, 38). We believe that soluble mediators released during FIP pathogenesis, e.g., by macrophages or neutrophils, are partly responsible for T-cell depletion through apoptosis. Thus, thymocytes and FIPV-specific T cells in the draining lymphoid organs (spleen and lymph nodes) and lesions (e.g., peritoneal cavity) may be killed before they can exert their antiviral activity. Several cytokines have been shown to induce apoptosis in activated lymphocytes (17, 37, 39). However, thus far we were unable to block the apoptosis-inducing factor in the conditioned media. Although both tumor necrosis factor alpha (TNF-α) and transforming growth factor β (TGF-β) were capable of inducing apoptosis in vitro and despite their production in situ, ascitic fluid and conditioned media did contain only low levels of the active cytokines. Moreover, using neutralizing antibodies against TNF-α and TGF-β we were not able

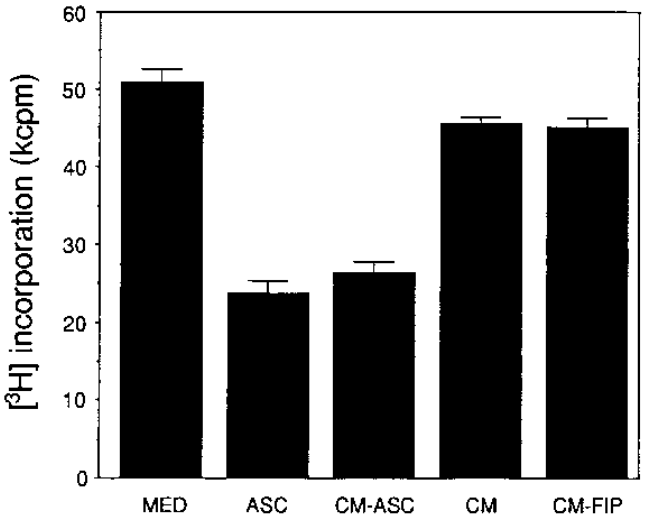

FIG. 4. Inhibition of T-cell proliferation by soluble mediators. Incorporation of thymidine was measured in feline thymocytes stimulated 3 days previously with ConA plus IL-2 in the presence of medium (MED), ascitic fluid (ASC), conditioned medium from peritoneal cells (CM-ASC), or mock-infected (CM) or FIPV-infected fcwf cells (CM-FIP).

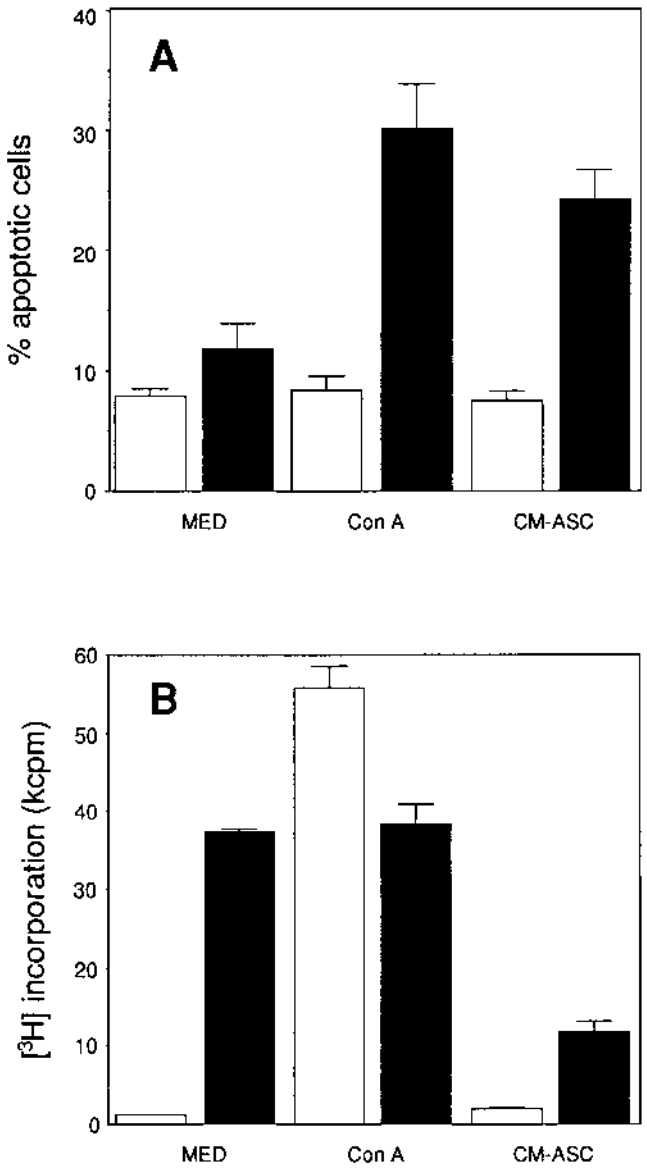

FIG. 5. Effect of conditioned medium from peritoneal cells of an FIP cat on T-cell apoptosis. Percentages of apoptotic cells as determined by in situ nick staining (A) and incorporation of thymidine (B) of ConA-activated T-cell blasts (solid bars) and nonactivated T cells (open bars) cultured in medium (MED), ConA, or CM-ASC.

to block apoptosis (9). This may indicate that other factors or mediators downstream of the TNF-α–TGF-β cascade are involved. We are currently analyzing the nature of the soluble mediator, its cellular origin, and its mechanism of induction. The cloning of feline cytokines (27, 30) and the development of specific reagents to inhibit feline cytokines should facilitate the study of the nature of the factor(s) involved.

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