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Δ^9-Tetrahydrocannabinol Induces Apoptosis in Macrophages and Lymphocytes: Involvement of Bcl-2 and Caspase-1[1]

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ABSTRACT

Apoptosis is programed cell death characterized by certain cellular changes and regulated by various gene products including Bcl-2 and caspase-1. The marijuana cannabinoid, Δ^9tetrahydrocannabinol (THC), has been reported to suppress in culture the proliferation of splenocytes and increase the release of IL-1 from macrophages; however, the mechanisms of these effects remain unclear. Because cannabinoids have also been reported to induce apoptosis and because the release of IL-1 and suppression of lymphoproliferation are related to apoptosis, we tested for the induction of apoptosis by THC in murine immune cell cultures. Splenocytes cultured with Con A for up to 24 hr showed evidence of DNA fragmentation determined by gel electrophoresis, terminal deoxynucleotide transferase-mediated dUTP-fluorescein nick end labeling and ^{3}H-thymidine labeling and THC (15–30 μM) treatment increased fragmentation under these conditions. Resident peritoneal macrophages cultured with lipopolysaccharides showed no obvious fragmentation unless they were also treated with THC. Time course studies examining DNA fragmentation and cell membrane integrity (assessed by dye exclusion) showed that fragmentation preceded membrane damage indicating that THC induced apoptosis rather than cell necrosis. In addition, THC treatment of splenocytes resulted in a decrease of Bcl-2 mRNA and protein as measured by Northern and Western blotting, respectively, and the drug induced apoptosis was blocked by the caspase inhibitor, Ac-Tyr-Val-Ala-L-aspartic acid aldehyde. These data suggest that THC treatment of cultured immune cells induces apoptosis through the regulation of Bcl-2 and caspase activity.

THC, the major psychoactive component of marijuana, has been shown to suppress immune functions, including lymphocyte proliferation, antibody production, natural killer activity and macrophage activity (Klein *et al.,* 1998). Previously we showed that THC increased IL-1 secretion and processing in resident peritoneal macrophages stimulated by LPS (Zhu *et al.,* 1994). The secretion and processing of IL-1 has been demonstrated to be associated with programed cell death, or apoptosis (Hogquist *et al.,* 1991), and recently, cannabinoids, were shown to induce apoptosis in human peripheral blood mononuclear cells (Schwarz *et al.,* 1994). Apoptosis is a process of cell death that occurs in response to a number of physiologically relevant stimuli. Cells undergoing apoptosis display several morphological and biochemical alterations, including reduced cell volume, condensed chromatin in the nucleus, organelle relocalization and the formation of internucleosomal DNA fragmentation (Wyllie, 1980; Wyllie *et al.,* 1980; Arends *et al.,* 1990; McConkey *et al.,* 1990). Although, the precise molecular mechanisms of apoptosis are unclear, recent data have implicated a number of gene products. One of these, Bcl-2, encodes a protein localized to intracellular membranes and originally cloned from the chromosomal breakpoint of the t (14;18) translocation present in many human B cell lymphomas (Tsujimoto *et al.,* 1984). Bcl-2 expression is widespread in a variety of tissues and cells, including thymocytes and peripheral lymphocytes (Veis *et al.,* 1993; Broome *et al.,* 1995). It has been demonstrated that Bcl-2 blocks apoptosis induced by diverse stimuli such as growth factor withdrawal, glucocorticoids, radiation and chemotherapeutic agents (Vaux *et al.,* 1988; Nunez *et al.,* 1990; Sentman *et al.,* 1991). However, the interleukin-1β converting enzyme now referred to as caspase-1 (Alnemri *et al.,* 1996), which is responsible for the proteolytic processing of IL-1β, appears to promote apoptosis (Black *et al.,* 1988; Kostura *et al.,* 1989; Thornberry and Molineaux, 1995). We demonstrate that treatment of macrophages and splenocytes with THC results in DNA fragmentation as well as suppression of Bcl-2 mRNA and protein. Furthermore, the drug-induced apoptosis was blocked by an inhibitor of caspase-1. These data suggest that the inhibitory effects of THC on *in*

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ABBREVIATIONS: THC, Δ^9-tetrahydrocannabinol; IL-1, interleukin-1; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-fluorescein nick end labeling; Con A, Concanavalin A; LPS, lipopolysaccharide; DMSO, dimethylsulfoxide; SDS, sodium dodecyl sulfate; PAGE, polyacrylacrylamide gel electrophoresis.

vitro immune responses might partially be caused by a drug induced apoptosis through an alteration of Bcl-2 and caspase-1 activity.

Methods

THC. THC was obtained from the National Institute on Drug Abuse (Rockville, MD) as a 98.6% tar. The drug was initially dissolved in DMSO (Sigma Chemical Co., St. Louis, MO) to a concentration of 200 mg/ml and stored under nitrogen gas at −20°C. For use, the stock drug was diluted (20 mg/ml) in DMSO and further diluted in warm tissue culture medium for addition to the cultures.

Cell preparation. Resident, peritoneal macrophages were obtained from BALB/c mice (The Jackson Laboratories, Bar Harbour, ME) by peritoneal lavage with Dulbecco's PBS phosphate-buffered saline (Sigma), washed in HBSS (Gibco, Grand Island, NY), and suspended in RPMI 1640 medium (Gibco) supplemented with 10% fetal calf serum (HyClone Labs, Logan, UT), L-glutamine, antibiotics and 2-mercaptoethanol (5×10^{-5} M). Peritoneal cells (10^7 cells/well) were incubated in six-well tissue culture plates for 2 to 3 hr followed by medium rinsing to enrich for adherent macrophages. Spleens were removed from BALB/c mice and single cell suspensions were prepared in HBSS in a Stomacher 80 Lab-Blender (Tekmar Co., Cincinnati, OH). The cells were then washed by centrifugation in HBSS and suspended in RPMI 1640 medium to final concentration of 2×10^6 cells/ml.

DNA preparation and electrophoresis. A modification of the procedure described by Hogquist *et al.* (1991) was used. Briefly, splenocytes (5×10^6 cells/well) were incubated for 2, 4, 6 and 24 hr in six-well plates with either THC (5–10 μg/ml) or DMSO (0.1%) and cotreated with the mitogen Con A (10 μg/ml; Sigma). Peritoneal macrophages (approximately 3×10^6 cells/well) were incubated for 2, 4 and 24 hr with either THC or DMSO in the presence of 10 μg/ml LPS (*Escherichia coli;* Sigma). In experiments involving the caspase-1 inhibitor (Ac-Tyr-Val-Ala-L-aspartic acid aldehyde; Bachem Bioscience, King of Prussia, PA), the inhibitor (150 μM) was added along with mitogens to the cultures at the start of the incubation. After incubation, splenocyte suspensions and macrophage cultures were washed with cold PBS and incubated on ice for 10 min in 0.5 ml of lysis buffer containing 20 mM Tris.HCl, pH 7.4, 10 mM EDTA and 0.2% Triton X-100. The lysates were centrifuged for 15 min at 12,000 × *g* and the supernatants incubated at 50°C overnight with proteinase K (0.1 mg/ml, Sigma) and then extracted with a 1:1 phenol/chloroform mixture. The DNA was precipitated at 20°C for 30 min with 1/5 volume 5 M ammonium acetate and 1 volume of isopropanol. After centrifugation, the samples were digested with 50 μg/ml RNase A for 1 hr at 37°C and the DNA concentration estimated by spectrophotometry. The DNA samples were loaded (20 μg/lane) into 1% agarose gels containing 1 μg/ml ethidium bromide and electrophoresed. For quantitation, films were scanned in a Bio-Rad (Hercules, CA), imaging densitometer, model GS-670 and the results reported in relative volume units.

DNA fragmentation assay. The radioisotope method of DNA fragmentation was used as previously described (Kamesaki *et al.,* 1994). In brief, splenocytes (10^7/ml) were incubated in culture tubes overnight with Con A and 2.5 μCi/ml ^{3}H-thymidine (2.0 Ci/mmol; ICN, Irving, CA). Labeled cells were washed three times with cold Dulbecco's phosphate-buffered saline and incubated further (5×10^6/well) in culture plates with Con A and either medium, THC or DMSO for 2, 4 and 24 hr. At the end of incubation, the cells were washed and lysed with lysis buffer as described above, centrifuged at 12,000 × *g* for 20 min, and the radioactivity in the supernatants and pellets determined by liquid scintillation counting. The percentage of DNA fragmentation was calculated using the following formula: % DNA fragmentation = CPM from supernatant/CPM from supernatant + CPM from pellet.

Fluorescent labeling of nuclear DNA fragments. DNA fragmentation *in situ* was determined by TUNEL using the *In Situ* Cell Death Detection kit (Boehringer Mannheim, Indianapolis, IN). Splenocyte cultures (5×10^6/ml) were incubated in supplemented RPMI 1640 medium in 6-well plates for 6 hr with Con A (10 μg/ml) and either DMSO or THC (10 μg/ml). After incubation, the cultures were washed twice with DPBS and processed for TUNEL according to the manufacture's instructions. This method is reported to be specific for apoptosis and in this method nuclear fluorescence (in the absence of other cellular changes) is shown to reach maximum levels in dexamethasone-treated thymocyte cultures at between 4 and 6 hr (Gavrieli *et al.,* 1992). The apoptotic cells were visualized using a fluorescent microscope equipped with a 35-mm camera system (Olympus, Tokyo, Japan).

Northern blot analysis. Splenocytes (1×10^7 cells/well) were incubated in six-well plates for 2 hr with Con A (10 μg/ml) and either medium, DMSO or THC (5 or 10 μg/ml). Total RNA was isolated using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH) and the concentration estimated by spectrophotometry. RNA samples (20 μl, 10 μg/lane) were loaded into 1% agarose gels after denaturation with glyoxal and DMSO and electrophoresed. The gels were then blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH) which were then baked and hybridized at 55°C for 2 hr using rapid hybridization buffer (Amersham Corp., Arlington Heights, IL). The cDNA probe used in these studies was amplified from mouse spleen RNA by RT-PCR using murine, Bcl-2 primers reported by Nunez *et al.* (1990). The PCR product was 865 bp and was labeled by the random-priming labeling system (Boehringer Mannheim). After hybridization, membranes were washed three times at room temperature in 2 × SSC (15 mM sodium chloride, 1.5 mM sodium citrate) containing 0.1% SDS followed by two washes at 55°C in 0.1 × SSC with 0.1% SDS. All membranes were stripped and rehybridized with γ actin cDNA as internal control. For quantitation, films were scanned in a Bio-Rad, imaging densitometer, model GS-670 and the results reported in relative volume units.

Western blot analysis. Splenocytes (1×10^7) were incubated with Con A (10 μg/ml) and either THC or DMSO for 2 hr. The splenocyte culture was then harvested, washed by centrifugation several times and the cells lysed in buffer containing 50 mM Tris.Cl, pH 6.8, 100 mM dithiothreitol, 2% SDS and 10% glycerol. Equal amounts of protein were loaded (20 μl/ml) and separated by 12% SDS-PAGE and then transferred to nitrocellulose. The blots were blocked in 5% milk for 1 hr. After incubation with hamster anti-mouse Bcl-2 antibody (Pharmingen, San Diego, CA) for 1 hr, a goat anti-hamster antibody conjugated with peroxidase (Accurate Chemical & Scientific Corp., Westbury, NY) was added for additional 1 hr. Blots were developed using enhanced chemiluminescence (Amersham). For quantitation, films were scanned in a Bio-Rad imaging densitometer, model GS-670 and the results reported in relative volume units.

Results

THC induces apoptosis. The hallmark of programed cell death is the formation of DNA fragments that are 180-bp multiples, which represent cuts between nucleosomes by the endonuclease activated in the process of apoptosis. Our previous data showed that THC increased the processing and release of IL-1 from macrophages (Zhu *et al.,* 1994) and the processing and release of this cytokine has been shown to be associated with apoptosis (Hogquist *et al.,* 1991). Thus, an initial goal was to determine whether or not THC was able to induce apoptosis in splenocytes and macrophages. Splenocytes were incubated for 2, 4, 6 and 24 hr with Con A and medium only or with Con A and either THC or DMSO. DNA was extracted and resolved by agarose gel electrophoresis. As shown in figure 1A, very little DNA fragmentation was observed in freshly isolated splenocytes (lane 1). However, frag-

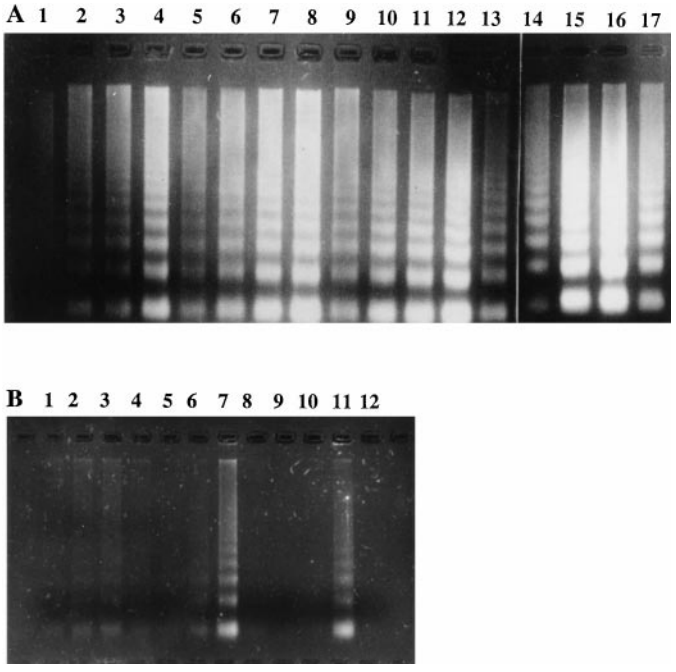

Fig. 1. THC treatment induces DNA fragmentation in splenocyte and macrophage cultures. Splenocytes (A) were incubated for various times with Con A (10 μg/ml) only, Con A and THC or Con A and DMSO. After incubation, DNA was extracted from cell lysates and electrophoresed (20 μg/lane) in agarose/ethidium bromide gels. Lane 1, Nonincubated cells. Incubated for 2 hr: lane 2, Con A only; lane 3, Con A and THC 5 μg/ml; lane 4, Con A and THC 10 μg/ml; lane 5, Con A and DMSO. Incubated for 4 hr: lane 6, Con A only; lane 7, Con A and THC 5 μg/ml; lane 8, Con A and THC 10 μg/ml; lane 9, Con A and DMSO. Incubated for 6 hr, lane 10, Con A only; lane 11, Con A and THC 5 μg/ml; lane 12, Con A and THC 10 μg/ml; lane 13, Con A and DMSO. Incubated for 24 hr: lane 14, Con A only; lane 15, Con A and THC 5 μg/ml; lane 16, Con A and THC 10 μg/ml; lane 17, Con A and DMSO. Macrophages (B) were incubated with LPS (10 μg/ml) only, LPS and THC or LPS and DMSO for various times followed by DNA extraction and electrophoresis. Cells incubated for 2 hr: lane 1, LPS only; lane 2, LPS and THC 5 μg/ml; lane 3, LPS and THC 10 μg/ml; lane 4, LPS and DMSO. Incubated for 4 hr: lane 5, LPS only; lane 6, LPS and THC 5 μg/ml; lane 7, LPS and THC 10 μg/ml; lane 8, LPS and DMSO. Incubated for 24 hr: lane 9, LPS only; lane 10, LPS and THC 5 μg/ml; lane 11, LPS and THC 10 μg/ml; lane 12, LPS and DMSO. The gels were also analyzed by densitometry with the following volume unit values: A, Lane 1, 0.018; lane 2, 0.023, lane 3, 0.042; lane 4, 0.48; lane 5, 0.18; lane 6, 0.56; lane 7, 1.18; lane 8, 1.76; lane 9, 0.53; lane 10, 1.21; lane 11, 1.28; lane 12, 1.62; lane 13, 0.23; lane 14, 0.026; lane 15, 1.46; lane 16, 1.96; lane 17, 0.42. B, Lane 1, 0.017; lane 2, 0.031; lane 3, 0.045; lane 4, 0.034; lane 5, 0.034; lane 6, 0.025; lane 7, 0.087; lane 8, 0.044; lane 9, 0.044; lane 10, 0.042; lane 11, 0.081; lane 12, 0.040. The results are representative of four experiments.

mentation increased with time of incubation (lanes 2, 6, 10 and 14) confirming the work of others that splenocytes spontaneously undergo apoptosis in culture. THC treatment further increased fragmentation at all four time points tested with the greatest increases occurring at the 24-hr time point. Peritoneal macrophage cultures were incubated for 2, 4 and 24 hr with LPS and either medium only, THC or DMSO. Figure 1B shows that, unlike splenocytes, fragmentation was not observed in macrophage cultures of up to 24 hr (lanes 1, 5 and 9). However, treatment with the highest concentration of THC (10 μg/ml) but not DMSO induced detectable fragmentation at the 4- and 24-hr incubation times (lanes 7 and 11). These DNA fragmentation studies suggested that THC increased apoptosis in both splenocyte and macrophage cultures.

Analysis of apoptosis by the TUNEL method. *In situ* DNA end labeling is a very sensitive indicator of apoptosis. To confirm the agarose gel electrophoresis data, we treated splenocytes with THC in the presence of Con A for 6 hr and then subjected the cells TUNEL to visualize cells with fragmented DNA in the nucleus (Gavrieli *et al.,* 1992). Figure 2A is a fluorescent micrograph of DMSO-treated cells and shows that control cells display a few apoptotic cells. This low level of apoptosis has been reported at 6 hr in cultured thymocytes (Gavrieli *et al.,* 1992). Figure 2B, however, shows that THC treatment greatly increased the number of cells containing peripheral nuclear fluorescence but no obvious other cellular changes. These results are similar to those observed in dexamethasone-treated cultures (Gavrieli *et al.,* 1992). The TUNEL results support the electrophoresis data and strongly suggest that THC treatment of leukocytes induces apoptosis.

DNA fragmentation precedes loss of membrane integrity. In these studies, DNA fragmentation was analyzed by yet another method (Kamesaki *et al.,* 1994). Splenocytes were prelabeled with ^{3}H-thymidine and then treated with several concentrations of THC for 2, 4 and 24 hr. The cpm in the cellular supernatants and pellets after lysing were counted and % DNA fragmentation was determined as described in "Methods." As seen in figure 3A, this radioisotope method showed results similar to the electrophoresis method

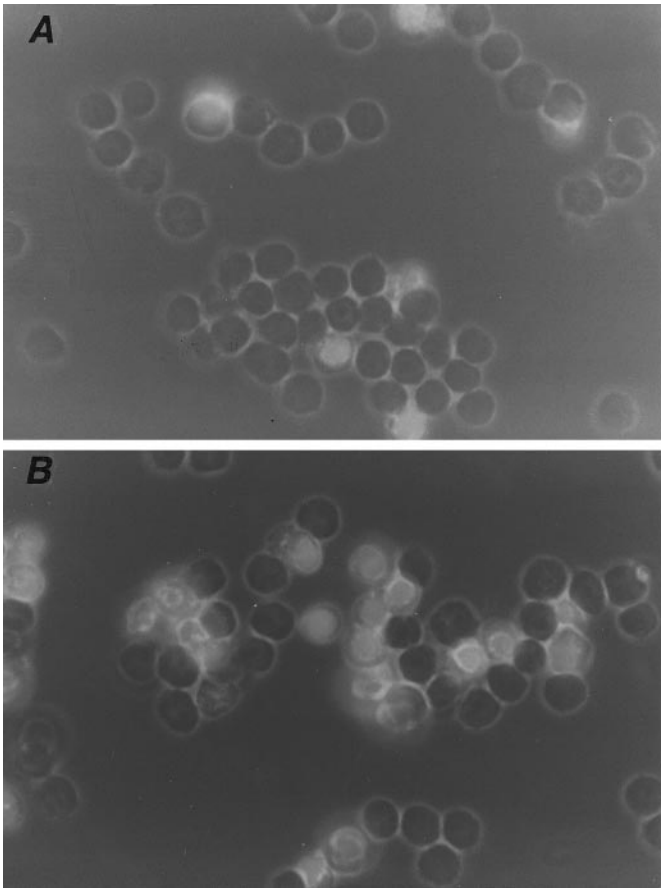

Fig. 2. THC induces nuclear DNA strand breaks as measured by TUNEL. Splenocytes were incubated for 6 hr with Con A (10 μg/ml) and either DMSO (0.1%; A) or THC (10 μg/ml; B). Cells were subsequently washed with buffer and processed for TUNEL according to the manufacturer's instructions and then viewed and photographed with the aid of a fluorescent microscope. Fluorescent micrograph in A shows a few cells labeled with the typical peripheral nuclear pattern. B shows THC treated cells and contains many more fluorescent cells than in the control cultures.

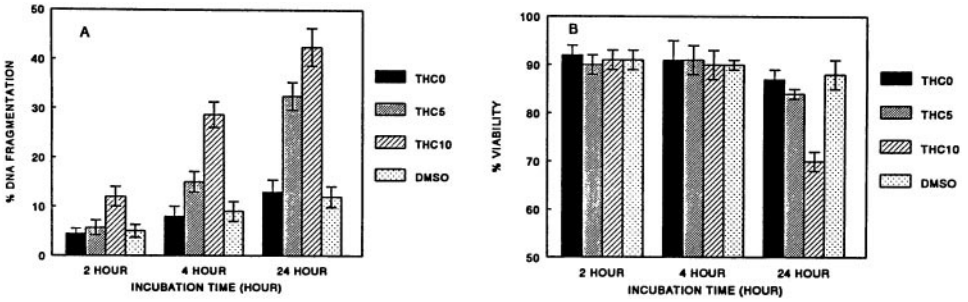

Fig. 3. THC induces DNA fragmentation in splenocytes before membrane damage. A, Splenocytes were pre-labeled with ^{3}H-thymidine and then incubated for 2, 4 and 24 hr with either THC or DMSO in the presence of Con A. After incubation, cells were lysed and the CPM determined in both pellet and supernatant fractions. The % DNA fragmentation was calculated as described in "Methods." B, Splenocytes incubated and treated as above and cell viability (membrane integrity) was determined by trypan blue staining. Data are reported as % viability (±S.E.M., four experiments) calculated from the number of cells taking-up the dye versus those excluding the dye. A total of 300 cells was counted per sample. Data are representative of four experiments.

above in that spontaneous DNA fragmentation occurred in untreated and DMSO treated cells after 2-hr incubation (about 5%) and reached 15% after 24-hr incubation, indicating that fragmentation increased with incubation time. THC treatment (5 μg/ml) increased the fragmentation over control at 4 and 24 hr and THC at the higher concentration of 10 μg/ml increased fragmentation at 2 hr and greatly increased fragmentation by 4 and 24 hr.

Because, after induction of apoptosis, DNA fragmentation precedes the loss of membrane integrity (Wyllie *et al.,* 1984; Arends *et al.,* 1990), we next examined the temporal relationship between loss of membrane integrity (trypan blue dye exclusion method) and DNA fragmentation. THC-treated cells excluded dye through 4 hr after treatment and even through 24 hr after the 5 μg/ml drug treatment (fig. 3B). However, cells treated with 10 μg/ml THC showed membrane damage at 24 hr suggesting the natural consequences of a more severe apoptosis. This suggested that the cell membrane was intact through 4 hr of drug-treatment, a time period shown above to display DNA fragmentation. Thus, DNA fragmentation in THC treated cells preceded the loss of membrane integrity and we therefore conclude the drug-induced apoptosis.

THC decreases the expression of Bcl-2 protein and mRNA. Bcl-2 is a protooncogene that blocks programed cell death. It has been demonstrated that splenocytes express readily detectable amounts of Bcl-2 protein (Broome *et al.,* 1995). To explore the possible role of Bcl-2 in the THC-induced apoptosis, we first examined the effects of THC on Bcl-2 protein expression. Splenocytes were incubated for 2 hr with Con A only or Con A plus either THC or DMSO. Cell lysates were prepared and the Bcl-2 protein analyzed by Western blotting. Consistent with other reports (Broome *et al.,* 1995), cultured, mitogen-stimulated, splenocytes expressed detectable levels of Bcl-2 protein (fig. 4, lane 1), and this was decreased by THC (10 μg/ml) treatment (fig. 4, lane 3). DMSO increased protein level (lane 4).

To determine whether the suppression of Bcl-2 protein is due to a drug-induced decrease in message, we next measured the level of Bcl-2 mRNA by Northern blotting. Splenocytes were treated with either THC or DMSO in the presence of Con A for 2 hr and the total RNA resolved by agarose gel electrophoresis, blotted and probed with ^{32}P-labeled, Bcl-2 cDNA. As shown in figure 5, a 7.5-kb transcript was detected (Nunez *et al.,* 1990). THC treatment (lanes 3 and 4) decreased the level of Bcl-2 mRNA relative to Con A only (lane 1) or Con A and DMSO (lane 2).

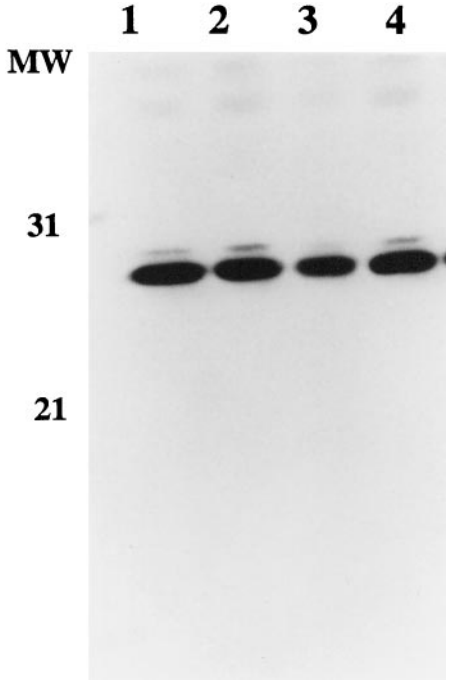

Fig. 4. THC treatment decreases Bcl-2 protein expression in splenocytes. Splenocytes were incubated for 2 hr with Con A (10 μg/ml) and either THC or DMSO. The cells were lysed and the protein extracts loaded (20 μl/lane) into SDS-PAGE gels for electrophoresis and Western blotting. Lane 1, Con A only; lane 2, Con A and THC 5 μg/ml; lane 3, Con A and THC 10 μg/ml; lane 4, Con A and DMSO. Densitometry readings in volume units were: lane 1, 111; lane 2, 136; lane 3, 90; lane 4, 148. Data are representative.

Caspase-1 inhibitor attenuates apoptosis induced by THC. The processing of promature forms of IL-1 to mature IL-1 requires caspase-1 which has been shown to play an important role in regulation of apoptosis (Thornberry and Molineaux, 1995). The reported increased IL-1β processing by THC (Zhu *et al.,* 1994) and the above results showing that THC induces apoptosis indicated that THC may affect caspase-1 activity. To test this, splenocyte and macrophage cultures were treated for 4 hr with Con A and LPS, respectively. As before, splenocytes showed some fragmentation after short-term incubation (fig. 6A, lane 1) although macro-

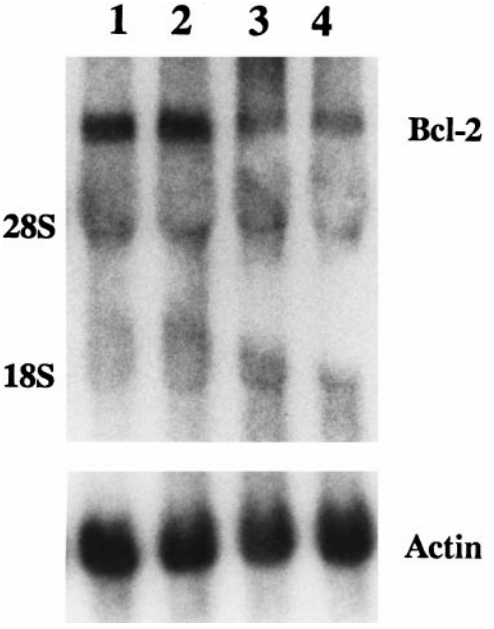

Fig. 5. THC treatment decreases Bcl-2 mRNA expression. Splenocytes were incubated for 2 hr with medium and Con A (10 μg/ml) only or Con A plus either THC or DMSO. Total RNA was extracted, denatured and loaded (20 μl/lane, 10 μg/lane) into 1% agarose gels, Northern blotted and probed with cDNAs for Bcl-2 and γ actin. Lane 1, Con A only; lane 2, Con A and DMSO only; lane 3, Con A and THC (5 μg/ml); lane 4, Con A and THC (10 μg/ml). Densitometry readings in volume units were: lane 1, Bcl-2-67, actin-144, ratio, 0.46; lane 2, Bcl-2-77, actin-151, ratio, 0.51; lane 3, Bcl-2-40, actin-124, ratio, 0.32; and lane 4, Bcl-2-30, actin-141, ratio, 0.21. Data are representative.

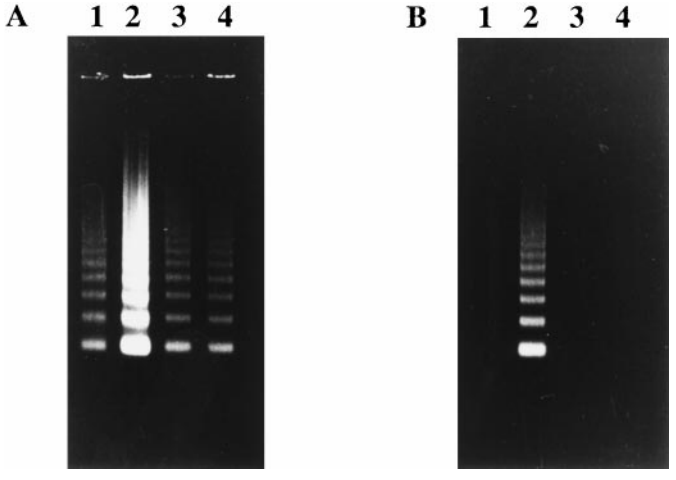

Fig. 6. Caspase-1 inhibitor, Ac-Tyr-Val-L-aspartic acid aldehyde, prevents DNA fragmentation induced by THC. Splenocytes (A) and macrophages (B) were incubated for 4 hr with Con A or LPS, with and without the inhibitor. Some cultures also received THC. DNA was extracted, resolved (20 μg/lane) on 1% agarose/ethidium bromide gels, and visualized for DNA laddering. A, Lane 1, Con A only without caspase-1 inhibitor; lane 2, Con A and THC (10 μg/ml) without inhibitor; lane 3, Con A only with caspase-1 inhibitor; lane 4, Con A and THC (10 μg/ml) with inhibitor. B, Lane 1, LPS only without inhibitor; lane 2, LPS and THC (10 μg/ml) without inhibitor; lane 3, LPS only with caspase-1 inhibitor; lane 4, LPS and THC (10 μg/ml) with inhibitor. Densitometry readings in volume units: A, lane 1, 2.07; lane 2, 27.6; lane 3, 2.25; lane 4, 1.36. B, lane 1, 0; lane 2, 1.34; lane 3, 0; lane 4, 0. Data are representative.

phages did not (fig. 6B, lane 1). Treatment with THC increased fragmentation in both cell types (fig. 6A and B, lane 2) but cotreatment with the caspase-1 inhibitor suppressed the drug effect in both cultures (fig. 6A and B, lane 4).

Discussion

THC modulates immune cell function, including the suppression of lymphocyte proliferation and the increased processing and release of IL-1 (Zhu *et al.,* 1994). Recently, the endogenous cannabinoid receptor ligand, anandamide, was shown to induce apoptosis in human lymphocyte cultures (Schwarz *et al.,* 1994). Because apoptosis has been related to suppression of proliferation (Lee *et al.,* 1993) and processing of IL-1 (Hogquist *et al.,* 1991), we hypothesized that apoptosis was occurring in THC-treated and mitogen-activated murine splenocyte cultures and LPS-activated murine macrophage cultures.

Apoptosis and necrosis are major modes of cell death (Schwartz and Osborne, 1993). Necrosis results from the application of noxious compounds and causes membrane injury resulting in rapid cell swelling and rupture. However, apoptosis requires the expression of new mRNA and protein and can occur under normal physiological conditions especially during developmental processes, or in response to various agents such as glucocorticoids, radiation and tumor necrosis factor. Apoptosis results in cell shrinkage, plasma membrane "boiling," nuclear chromatin condensation, DNA fragmentation and repackaging of the cell into smaller apoptotic bodies (Schwartz and Osborne, 1993, Cohen *et al.,* 1992, Cory, 1995). Of these, DNA fragmentation is widely used to assess apoptosis. In this study, we used several measures of DNA fragmentation. First, it was demonstrated by agarose gel electrophoresis (fig. 1) and the characteristic laddering of DNA due to intranucleosomal cleavage was observed. Con A-stimulated splenocytes showed low but detectable levels of fragmentation through 24 hr of culture. This low level of fragmentation has been reported in other types of immune cell cultures (Gavrieli *et al.,* 1992, Schwarz *et al.,* 1994). Basal fragmentation, however, was increased by THC treatment with notable changes occurring as early as 4 hr. Cultured resident peritoneal macrophages stimulated with LPS did not display spontaneous apoptosis but as with splenocytes could be induced to fragmentation by THC treatment. DNA fragmentation was also demonstrated by the TUNEL method that preferentially labels DNA strand breaks generated in the nucleus during apoptosis and differentiates apoptosis from necrosis. As with electrophoresis analysis, the TUNEL method showed that cultured splenocytes had a basal level of DNA breaks in the nucleus but that THC treatment increased the number of these cells (fig. 2). It should be noted that the TUNEL positive cells are of normal size and not swollen as would be expected in cells undergoing necrosis. Also, the fluorescent pattern was perinuclear, again typical of apoptosis rather than necrosis (Gavrieli *et al.,* 1992).

That the cells were undergoing an increase in apoptosis rather than necrosis is supported by additional findings. Apoptosis and necrosis differ in that within hours after the administration of the death signal the plasma membrane in apoptosis is left intact and capable of excluding vital dyes (Cohen *et al.,* 1992). This is not the case after the administration of a noxious agent that induces necrosis wherein the cell membrane is damaged within minutes and vital dyes are not excluded. In our studies, membrane damage was evident in less than 10% of splenocytes as determined by trypan blue exclusion through the first 4 hr of incubation in both control

and drug treated cultures. However, DNA fragmentation was observed at both 2 and 4 hr of THC treatment (fig. 3). At 24 hr, control cultures and even THC-treated (5 μg/ml) cultures still showed almost 90% viable cells, despite the fact that the drug treated cultures showed augmented DNA fragmentation. The higher drug concentration (10 μg/ml), however, caused at 24 hr substantial fragmentation and also loss of membrane integrity suggesting that THC at this time point and concentration was either inducing membrane breakdown subsequent to apoptosis or inducing a combination of apoptosis and necrosis.

The above results suggest that THC treatment of Con A-activated splenocytes and LPS-activated macrophages increased the apoptotic activity of the cultures. Many genes participate in the regulation of apoptosis (Schwartz and Osborne, 1993; Cory, 1995). These genes can be classified into those primarily suppressing apoptosis, such as the Bcl-2 gene family, and those facilitating apoptosis, such as members of the caspase family. To determine if these proteins were involved in THC-induced apoptosis, we next examined the effect of drug treatment on the expression of Bcl-2. We observed that murine splenocytes stimulated for 2 hr with Con A expressed appreciable amounts of Bcl-2 protein and mRNA (figs. 4 and 5); however, both of these levels were suppressed by THC treatment. Thus, there was an inverse relationship between the level of Bcl-2 (lower) and the degree of apoptosis (higher) as suggested by others (Cory, 1995; Broome *et al.,* 1995). Although the mechanism by which Bcl-2 inhibits apoptosis is not known, there is evidence suggesting that the level of Bcl-2 mRNA and protein is regulated at the level of transcription via several negative regulatory elements that bind in the 5′-untranslated region of the Bcl-2 gene (Cory, 1995; Young and Korsmeyer, 1993). It is possible that THC binding to cannabinoid receptors might activate these negative regulators and downregulate Bcl-2 because cannabinoid receptors are reported to be expressed on immune cells (Kaminski *et al.,* 1992) and these receptors have been linked to gene signaling factors such as adenylyl cyclase (Howlett *et al.,* 1988), mitogen-activated protein kinase (Bouaboula *et al.,* 1995b), Krox-24 (Bouaboula *et al.,* 1995a) and NFκB (Daaka *et al.,* 1997). However, we were unable to show a structure/activity relationship indicative of cannabinoid receptor involvement (unpublished) and the precise role of transcription factors such as NFκB in the regulation of apoptosis is currently unclear (Kolberg, 1997).

In addition to Bcl-2, drug-induced changes in the activity caspase-1 might also contribute to the induction of apoptosis. Caspase-1 is responsible for proteolytic processing of premature IL-1β to mature IL-1β and, as mentioned above, is also of major importance in apoptosis (Nicholson, 1996). For example, overexpression of caspase results in apoptosis, and this can be blocked by either Bcl-2 or caspase inhibitors (Thornberry and Molineaux, 1995). Because we had shown that THC suppressed proliferation, augmented IL-1 processing and induced apoptosis in splenocytes and macrophages (see above), we hypothesized that the caspase inhibitor Ac-Tyr-Val-Ala-L-aspartic acid aldehyde might suppress the THC effect on DNA fragmentation. In fact, this was observed in both splenocyte and macrophage cultures (fig. 6) supporting the view that THC induces apoptosis in these cells. It is not clear at this time how THC might be affecting the caspase activity and whether or not this is mediated by cannabinoid receptors. It does appear clear, however, that THC treatment of splenocyte and macrophage cultures can induce apoptosis by molecular pathways established for other environmental triggers such as heat shock and glucocorticoids (Cory, 1995), and that this induction of apoptosis may be the basis for some of the immunomodulating effects observed in cell culture models.

Whether or not apoptosis occurs *in vivo* after cannabinoid exposure is not addressed by our studies. Certainly, it is not likely that in marijuana smokers, the blood entering the spleen contains THC in the concentrations used in our studies (10–30 μM), although heavy marijuana abusers can use up to 20 mg/kg/day (Nahas *et al.,* 1977). However, regarding *in vivo* effects, it should be kept in mind that spleen and lymph nodes are reported to be exceptional among peripheral tissues in the density of cannabinoid specific binding sites, suggesting that these cells may have a heightened sensitivity to cannabinoids either ingested or produced locally from arachidonic acid (Lynn and Herkenham, 1994). However, as mentioned above, we could find no evidence of cannabinoid receptor involvement in our studies using cultured lymphoid cells. Further experiments are needed to more fully understand the molecular mechanisms involved in THC-induced apoptosis and the *in vivo* relevance of our observations.

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