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International Immunopharmacology 3 (2003) 1889–1900

International Immunopharmacology

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Preliminary report

An extract of *Uncaria tomentosa* inhibiting cell division and NF-κB activity without inducing cell death

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Received 6 May 2003; received in revised form 26 June 2003; accepted 24 July 2003

Abstract

Previous reports have demonstrated that extracts of the plant *Uncaria tomentosa* inhibit tumor cell proliferation and inflammatory responses. We have confirmed that C-Med 100®, a hot water extract of this plant, inhibits tumor cell proliferation albeit with variable efficiency. We extend these findings by showing that this extract also inhibits proliferation of normal mouse T and B lymphocytes and that the inhibition is not caused by toxicity or by induction of apoptosis. Further, the extract did not interfere with IL-2 production nor IL-2 receptor signaling. Since there was no discrete cell cycle block in C-Med 100®-treated cells, we propose that retarded cell cycle progression caused the inhibition of proliferation. Collectively, these data suggested interference with a common pathway controlling cell growth and cell cycle progression. Indeed, we provide direct evidence that C-Med 100® inhibits nuclear factor κB (NF-κB) activity and propose that this at least partially causes the inhibition of proliferation.
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Keywords: *Uncaria tomentosa*; Proliferation; Apoptosis; NF-κB; Inhibition

1. Introduction

Hot tea from the shredded bark of the vine *Uncaria tomentosa* has been used for generations by the Asháninka Indians in Peru as a drug against disorders like inflammation, cancer and infections [1,2]. Already in 1997, more than 50 dietary supplement manufacturers in the US offered products containing extracts from this plant, which exposes its commercial interest [3]. Numerous reports have shown that extracts from *U. tomentosa* possess not only anti-inflammatory activity, but also anti-viral, anti-mutagenic and anti-oxidant activities [4–9]. Furthermore, such extracts have been reported to enhance phagocytosis [10] and to stimulate the production of IL-1 and IL-6 in rat alveolar macrophages [11]. Interestingly, the extracts also inhibit the proliferation of certain cancer cells [12] and function as a non-competitive inhibitor of estradiol binding in hormone-dependent tumors [13].

Most importantly, extracts from cat's claw inhibit TNFα production [14] and inhibit the activation of the

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doi:10.1016/j.intimp.2003.07.001

transcription factor nuclear factor κB (NF-κB) [15]. These findings suggest that extracts from *U. tomentosa* mediate their anti-inflammatory effect through inhibition of NF-κB and thereby the synthesis of the important pro-inflammatory cytokine TNFα. NF-κB is activated by several different signals [16] and constitutes a family of transcription factors that is important for cellular functions such as cell cycle progression and proliferation (reviewed in Refs. [17,18]). NF-κB has also been implicated in inflammatory diseases such as rheumatoid arthritis, multiple sclerosis, asthma, inflammatory bowel disease and atherosclerosis (reviewed in Refs. [19,20]). This factor also regulates the expression of pro-inflammatory cytokines like TNFα, IL-1, IL-2, IL-6 and IL-8 (reviewed in Ref. [19]) and is involved in the regulation of apoptosis (reviewed in Ref. [21]). Components in *U. tomentosa* extracts [15] that are able to inhibit or alter the effects of NF-κB are therefore potential candidates for the development of novel pharmaceutical anti-inflammatory agents.

C-Med 100® is an aqueous extract from the bark of *U. tomentosa* that is derived through water extraction and separation of higher molecular weight components such as tannins and only contains molecules with a molecular weight < 10 kDa [22,23]. This extract has been shown to induce apoptosis in some human leukemic cell lines [24] as well as to enhance DNA repair [22,25]. Further, Lamm et al. [26] supplemented humans with C-Med 100® during vaccination and showed a prolonged antibody response to a pneumococcal vaccine in the C-Med 100®-treated patients. Sheng et al. [23] previously reported that the C-Med 100® extract also significantly accelerated the recovery of white blood cells (WBC) in rats treated with the chemotherapeutic agent doxorubicin. Similarly, supplementation with C-Med 100® lead to an elevated number of WBC in both rats and humans [22]. We have recently confirmed that observation in the mouse [27]. We also showed that the elevated cell number was partly due to prolonged lymphocyte survival and cell numbers returned to normal level within a month after withdrawal of the C-Med 100® extract [27].

In this paper, we have further analysed the impact of C-Med 100® on cell growth in vitro and its molecular mechanism of action. We show that the C-Med 100® extract inhibited proliferation in both cell lines and primary lymphocytes without induction of apoptosis. Furthermore, we report that this extract also inhibited the activity of the transcription factor NF-κB, which is involved in both lymphocyte activation and proliferation.

2. Material and methods

2.1. Mice

C57BL/6 females were bought from M&B, Ry, Denmark and used in experiments at an age of 6–10 weeks. The animals were kept in a SPF facility at Lund University. The use of laboratory animals complies with the guidelines of the European Community and was approved of by the local ethical committee.

2.2. C-Med 100®

C-Med 100® is a patented extract from *U. tomentosa*, Cat's claw (U.S. patent 6,039,949) supplied by CampaMed (New York, NY, USA). The extract is water-soluble and ultra-filtered to remove high molecular weight (MW) conjugates (>10,000 Da). The extract contains carboxy-alkyl-esters (CAE) as active ingredients (8–10%) and is almost free of oxindole alkaloids [23]. The active (CAE) components have indeed been identified as Benzoic acid analogs such as Quinic acid (U.S. patent application EL781388471US, filed March 7 2002 and Åkesson et al., unpublished data). The extract contains no detectable gram negative bacteria and endotoxin. It was dissolved in RPMI medium 30 min before use.

2.3. Fluorochrome-conjugated reagents

The following reagents were used for flow cytometry analysis: Fluorescein isothiocyanate (FITC)-conjugated Annexin V (Molecular Probes, Leiden, Holland), anti-Igκ (HB58) (prepared in our laboratory). Phycoerythrine (PE)-conjugated anti-CD4 (RM4-5), anti-CD8α (53–6.7), Annexin V (Pharmingen, San Diego, CA). Peridinin chlorophyll protein (PerCP)-conjugated anti-CD8α (53–6.7) (Pharmingen) and Cyanin 5 (Cy-5)-conjugated anti-B220 (RA3.6B2) (prepared in our laboratory).

2.4. Flow cytometry

Cells were counted and aliquots of 10^6 cells were stained with monoclonal antibodies in FACS-buffer [HBSS supplemented with 0.1% (NaN_3) and 3% fetal calf serum (FCS) (Life Technologies, Paisley, GB)], as previously described [27]. Spleen cells were preincubated for 10 min on ice with the anti-Fc receptor antibody 2.4G2 (Fcγ III/II) (prepared in our laboratory) to prevent unspecific binding to Fc-receptors [28]. The cells were analysed with a FACSCalibur flow cytometer (Becton Dickinson, San José, CA).

2.5. Cell lines and culture conditions

The HL60 human lymphoma (CCL-240), Raji human Burkitt's lymphoma (CCL-86), 70Z/3 mouse pre-B lymphocyte cell line (TIB-158), Jurkat human acute T-cell leukaemia (TIB 152) and mouse CTLL-2 cells or mouse spleen cells were used in the experiments. The cells were cultured in RPMI medium (Life Technologies) supplemented with 10% FCS, 10 mM HEPES buffer, antibiotics, 50 μM 2-mercaptoetanol and 1 mM sodium pyruvate (all supplements from Life Technologies) at 37 °C, 5% CO_2. Cells were stained with 2 μg/ml 7-amino-actinomycin D (7AAD; Sigma-Aldrich, St. Louis, MO, USA) and an optimal concentration (according to the manufactures protocol) Annexin V (Molecular Probes) and the cells were defined as apoptotic (Annexin V^+ $7AAD^-$) or dead ($7AAD^+$) by flow cytometry. For cell cycle analysis, Raji cells were labeled with propidium iodide [29] by incubation for 20 min with Vindelövs low salt solution [3.4 mM Tris–HCl (pH 7.6), 75 μM propidium iodide, 0.1% NP40, 10 mM NaCl]. The cells were subsequently analysed by flow cytometry. Spleen cells were polyclonally activated with 2.5 μg/ml concanavalin A (Con A; Amersham Pharmacia, Uppsala, Sweden), 10 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich), or 1 μg/ml anti-CD3 antibody (145.2C11; prepared in our laboratory). PMA (50 ng/ml), Ionomycin (1 μM), Cyclosporin A (1 μg/ml) (all from Sigma-Aldrich) and Pyrollidine dithiocarbamate (PDTC) (100μM) (EMD Bioscience, Calbiochem, San Diego, CA, USA) were used in some cultures as indicated. CTLL-2 cells were starved from IL-2 (2% supernatant from X63-IL-2 transfected cell line) for 4 h before they were re-cultured for 22 h. Proliferation was detected by measuring thymidine incorporation after a 4-h pulse with 1 μCi ^{3}[H]-thymidine (Amersham Pharmacia). To study cell division rate, spleen cells were stained with 1 μM carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes) in PBS. The cells were washed once in PBS before they were incubated with 1 ml CFSE for every 10^7 cells for 8 min at room temperature in the dark. To stop the reaction, one volume of FCS was added to the cells before they were washed twice in complete culture medium, activated and analysed by flow cytometry.

2.6. Assay for IL-2

The production of IL-2 in culture supernatants was investigated using an IL-2 specific ELISA (Pharmingen) according to the protocol from the manufacturer.

2.7. Preparation of cellular extracts

Whole cell extracts for immunoprecipitation and Western blot were prepared from 5×10^6 CTLL-2 cells after 4 h of IL-2 starvation and 2-h preincubation with C-Med 100®. The cells were stimulated with IL-2 as above for various time points at 37 °C whereafter, they were centrifuged at 3000 rpm for 2 min. The pellet was dissolved in 500 μl lysis buffer [20 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, 3 mM EGTA, 1% Nonidet P-40 containing the following inhibitors 5 mM *p*-nitrophenylphosphate, 10 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 5 mM NaF, and a proteinase inhibitor cocktail (Roche Diagnostics Scandinavia, Bromma, Sweden)] and the cells were lysed on ice for 30 min. The extracts were centrifuged at 14,000 rpm for 10 min at 4 °C and the supernatants were transferred to new tubes. The samples were stored at − 70 °C until immunoprecipitation was performed.

Whole cell extracts from LPS-stimulated 70Z/3 cells (1×10^6) were prepared for analysis of the NF-κB signaling pathway. The cells were washed twice in PBS, resuspended in lysis buffer [75 mM Tris–HCl (pH 8.0), 100 mM NaCl, 5 mM KCl, 3 mM $MgCl_2$, 2% NP-40, 1 mM PMSF and a proteinase inhibitor cocktail (Roche Diagnostics Scandinavia)] and incu-

bated on ice for 10 min. The cell debris was pelleted and the supernatants were stored at -70 °C until Western blot was performed.

2.8. Immunoprecipitation and Western blotting

The CTLL-2 cell lysates were precleared with 50 μl/sample of protein A sepharose beads (Amersham Pharmacia) by rotating for 1 h at 4 °C whereafter the beads were spun down and the supernatants were transferred to fresh tubes. Polyclonal anti-STAT5B antibodies (5 μl/10^7 cells) (Santa Cruz Biotechnology, Santa Cruz, CA) were added to the precleared samples. The samples were rotated for 1 h at 4 °C whereafter 50 μl/sample protein A sepharose beads (Amersham Pharmacia) were added for another 2 h. The immunoprecipitated proteins were pelleted and washed three times in lysis buffer. The proteins boiled in SDS-sample buffer for 5 min at 95 °C and then loaded on a 10% SDS-polyacrylamide gel. The separated proteins were transferred to a nylon membrane Hybond-C Extra (Amersham Pharmacia) by semidry blotting. The membranes were blocked for 1 h at room temperature in 5% dry fat-free milk in TBST [10 mM Tris–HCl (pH 8), 150 mM NaCl, 0,05% Tween-20]. STAT proteins were detected by incubation with 1 μg/ml monoclonal anti-STAT5 antibody (Transduction Laboratories, Lexington, KY) or 1 μg/ml anti-phosphotyrosine-STAT5 antibody (New England Biolabs, Beverly, MA) at 4 °C overnight. The membranes were subsequently washed three times with TBST and incubated with 100 pg/ml anti-mouse IgG (conjugated HRP) (Santa Cruz Biotechnology) in TBST for 1 h. The membranes were washed three times and chemoluminescence was detected using ECL-reagent (Amersham Pharmacia) and X-ray film (CEA, Strängnäs, Sweden).

Cellular extracts from 70Z/3 cells were separated on a 10% SDS polyacrylamide gel and the proteins were transferred to a nylon membrane. After blocking overnight in 5% dry fat-free milk in TBST, incubation with a primary anti-IκBα antibody (diluted 1:1000) (Cell Signaling Technology, Beverly, MA) for 2 h was performed. The membranes were washed three times in TBST, and incubated with an anti-rabbit antibody (diluted 1:4000) (conjugated with HRP) (Amersham Pharmacia). Chemoluminescence was detected as above.

2.9. Transient transfection and luciferase activity analysis

The reporter construct containing NF-κB binding sequences and the luciferase reporter gene was previously described [30]. Jurkat T cells were transiently transfected with the construct using the lipofectin method as described by the manufacturer (Life Technologies). Briefly, 2 μg reporter plasmid was mixed with 10 μl lipofectin and added to 3×10^6 Jurkat cells. The cells were rested for 22 h, pooled and pre-cultured for 2 h in the presence or absence of C-Med 100® before stimulation. After 6 h of stimulation, the cells were harvested, washed twice in phosphate-buffered saline (PBS) and treated with 100 μl Reporter Lysis Buffer according to the manufacturers recommendations (Promega, Madison, WI). Twenty microliters of each lysate was assayed for luminescence with Luciferase Assay Substrate (Promega) in a MicroLumat LB 96 P luminometer (EG&G Berthold, Wallac Sverige, Upplands Väsby, Sweden).

2.10. Statistics

Statistical analysis was performed using Student's two tailed *t*-test for unequal variance.

3. Results

3.1. C-Med 100® inhibits tumor cell proliferation but does not induce cell death

Previous data have demonstrated that the C-Med 100® extract inhibits proliferation and in parallel induces apoptosis in the HL60 tumor cell line while other tumor cells were relatively resistant to apoptosis induction [24]. We also failed to detect significant induction of cell death in other C-Med 100®-treated tumor cell lines at concentrations that still inhibited proliferation. Thus, using three different human tumor cell lines and a mouse pre-B cell line, we observed a dose-dependent inhibition of cell growth in the presence of C-Med 100® (Fig. 1A) compared to control cultures. The growth of Raji and HL60 cells was more profoundly inhibited than Jurkat cells (Fig. 1A), while the growth of 70Z/3 cells was only marginally inhibited at the highest

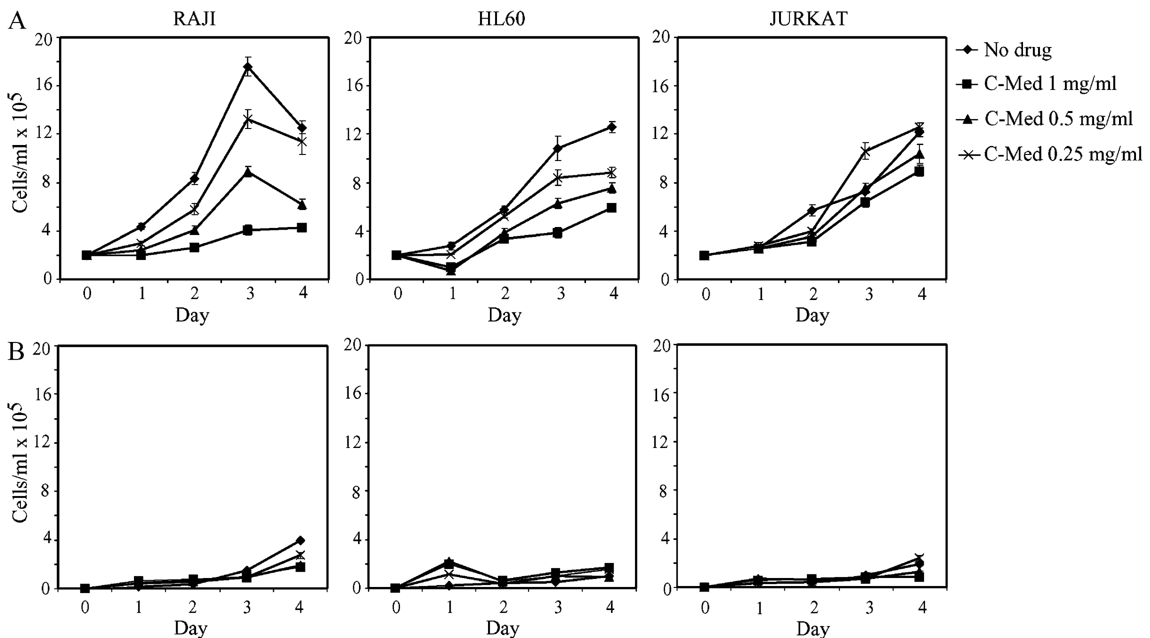

Fig. 1. Dose-dependent inhibition of proliferation in C-Med 100®-treated cell lines. 2×10^5 Raji, HL60 or Jurkat cells were cultured in duplicate in the absence or presence of indicated concentrations of C-Med 100®. After indicated time of culture, the cells were stained with trypan blue and both the numbers of viable (A) and (B) dead cells were counted. The data show the mean number of viable or dead cells/ml ± S.D. from one out of two similar experiments.

concentration of C-Med 100® (data not shown). However, there was no significant increase in the absolute number of dead cells at any of the C-Med 100® concentrations compared to the control cultures (Fig. 1B). Together, these data indicate that C-Med 100® treatment with variable efficiency inhibits growth of these cell lines in vitro but without inducing significant cell death.

3.2. C-Med 100® inhibits proliferation of primary lymphocytes

The observation that C-Med 100® inhibited the growth of tumor cell lines with variable efficiency prompted us to investigate whether this extract would also interfere with proliferation of ex vivo lymphocytes. Mouse spleen cells were therefore activated with T and B cell mitogens in the presence of C-Med 100® and the proliferation responses measured. Proliferation induced by either of the T cell mitogens anti-CD3 or Con A was inhibited in a dose-dependent way (Fig. 2A). Proliferation induced by the B cell mitogen LPS was also inhibited (Fig. 2A), but higher concentrations of C-Med 100® were required to achieve the same extent of inhibition. Thus, C-Med 100® inhibited mitogen-induced proliferation of both primary T cells and B cells. Taken together with the above data, these results suggested that C-Med 100® might interfere with some general mechanisms controlling cell cycle progression. However, mitogen-induced proliferation could potentially be blocked at several other levels as well.

To further explore the possible levels of inhibition of mitogen-induced proliferation, we stained mouse lymphocytes with CFSE and stimulated them in vitro with Con A or LPS as above. The fraction of cells that had divided was subsequently determined by flow cytometry. The results showed that a larger fraction of the $CD4^+$ T cells grown in the presence of C-Med 100® had not divided after 2 days of culture (Fig. 2B). Further, fewer of the cells that had divided once progressed through subsequent divisions. Analysis of $CD8^+$ T cell division in the same cultures gave similar results (data not shown). Thus, C-Med 100® detectably decreased the fraction of T cells entering mitosis. Conversely, LPS-stimulated B cells divided identical-

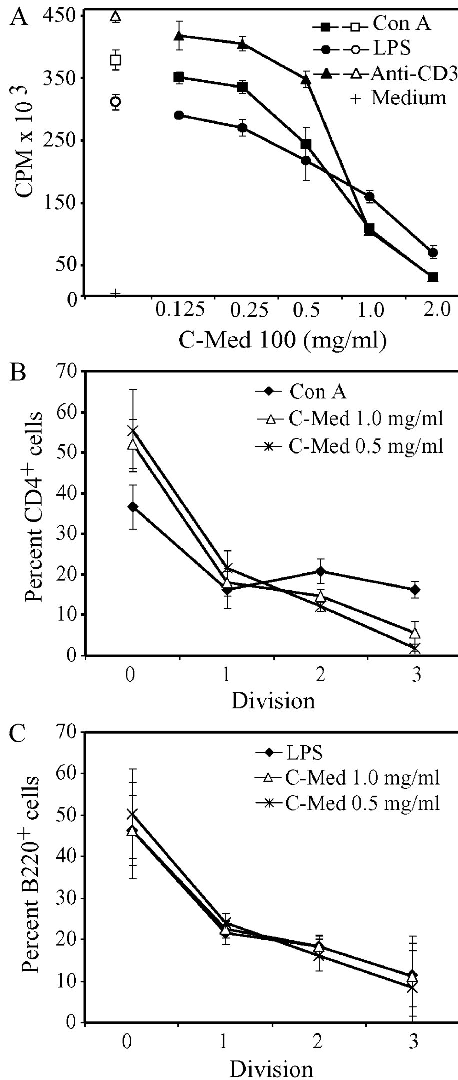

Fig. 2. (A) C-Med 100® inhibits proliferation of mitogen-stimulated lymphocytes. Spleen cells (2×10^5) were activated either with Con A, anti-CD3 antibodies or LPS in the presence of various concentrations of C-Med 100®. Proliferation was assayed after 48 h. One representative experiment out of three is shown as mean values ± S.D. (B and C) Spleen cells (1×10^6) were stained with CFSE and stimulated with Con A or LPS in the presence of C-Med 100®. After 48 h, duplicate cultures were stained with CD4, CD8 and B220 antibodies and analysed by flow cytometry. The percentage of dividing and non-dividing $CD4^+$ or $B220^+$ cells was determined. The data are pooled from two similar experiments and shown as mean values ± S.D.

ly in the presence and absence of C-Med 100® concentrations (Fig. 2C) that still efficiently inhibited proliferation (Fig. 2A).

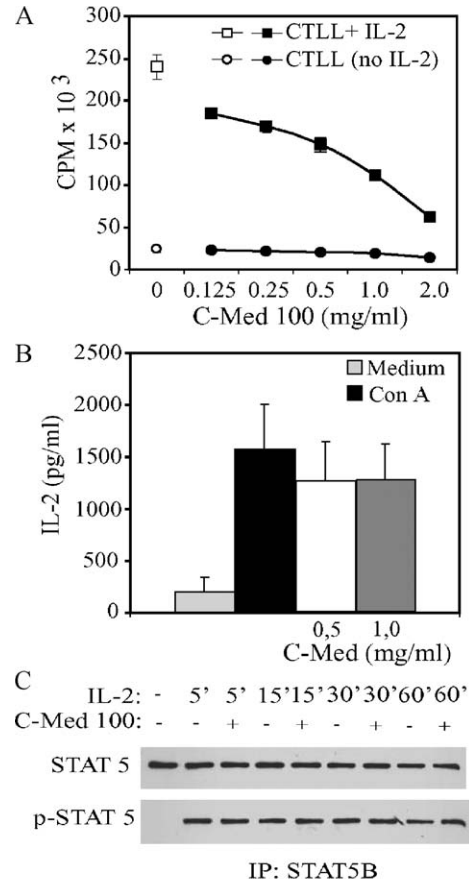

Fig. 3. (A) C-Med 100® inhibits proliferation of IL-2-stimulated CTLL-2 cells. CTLL-2 cells (5×10^3) were cultured in the presence of IL-2 and C-Med 100® and proliferation was assayed after 18 h by ^{3}H-thymidine incorporation in triplicate cultures. One representative experiment out of three is presented and shown as mean values ± S.D. (B) C-Med 100® does not inhibit the production of IL-2. Spleen cells (5×10^6) were polyclonally activated with Con A (2.5 μg/ml) in the presence of various concentrations of C-Med 100®. After 24 h, the supernatants were collected and the IL-2 content was determined with ELISA. The data are pooled from six similar experiments and shown as mean values ± S.D. (C) Expression of STAT5 and phospho-STAT5 in CTLL-2 cells. CTLL-2 cells were grown in medium for 2 h and then during the last 2 h of starvation some cultures were cultured in 1 mg/ml of C-Med 100® for another 2 h. The cells were then re-cultured (5×10^6 cells/culture) in medium supplemented with IL-2. The cultures were lysed at various time points as indicated and analysed by Western blotting. One representative experiment out of two is presented.

We also explored the possibility that C-Med 100® might interfere with IL-2 production and IL-2 induced proliferation in the T cell mitogen-stimulated cultures. Whereas IL-2 induced T cell proliferation was inhibited in a dose-dependent way (Fig. 3A), IL-2 production was not inhibited (Fig. 3B). Further, the inhibition in proliferation was most likely not due to deficient IL-2-receptor signalling as Stat-5 phosphorylation was identical in control and C-Med 100®-incubated T cells responding to IL-2 (Fig. 3C). Taken

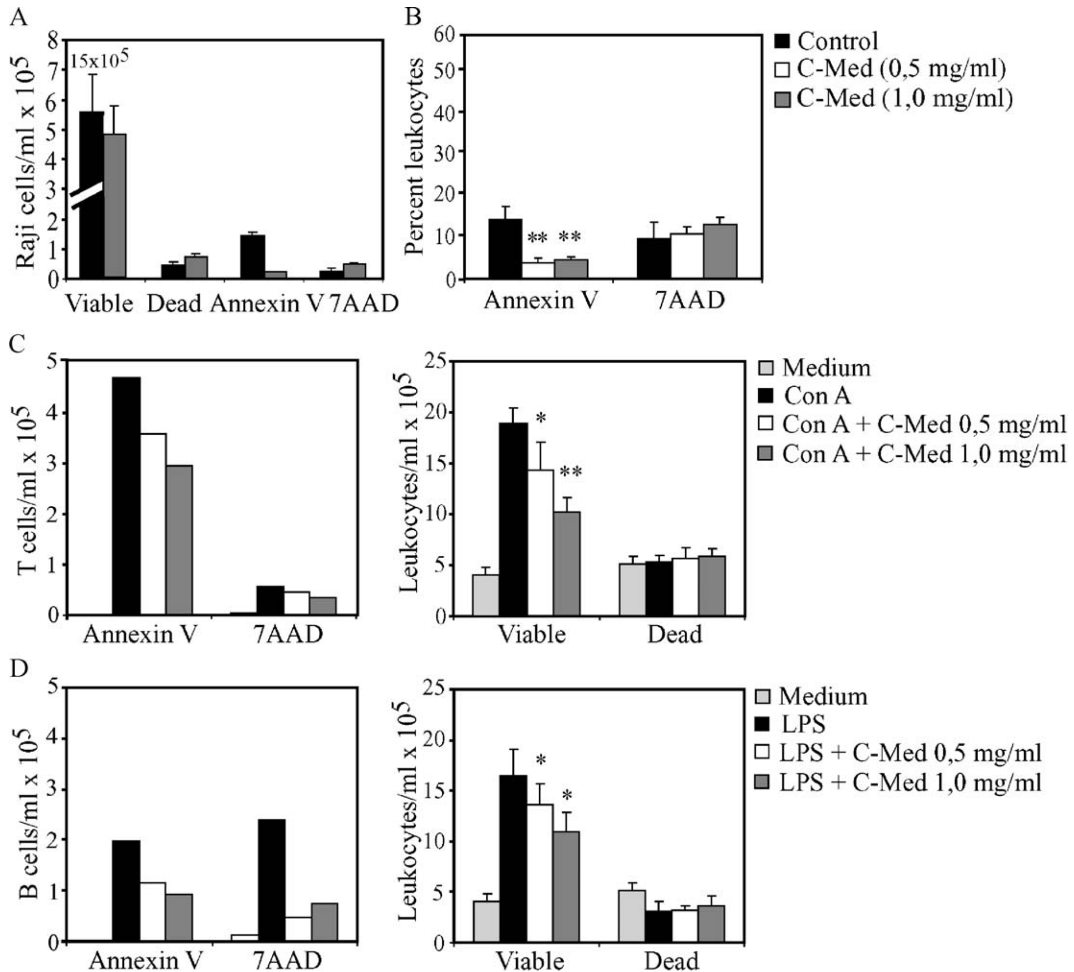

Fig. 4. (A) C-Med 100® induces no cell death at concentrations inhibiting proliferation. Raji cells (2×10^5) were cultured for 48 h in the presence of various concentrations of C-Med 100® and thereafter stained with Annexin V and 7AAD before analysis by flow cytometry. The results are presented as absolute number Annexin V^+ $7AAD^-$ (apoptotic) or $7AAD^+$ (dead) cells. The data are the mean values ± S.D. of duplicates from two similar experiments. (B) Spleen cells (1×10^6) were grown in medium alone for 48 h in duplicate cultures and analysed as in (A). The data shown as a percentage of control cultures are pooled from three independent experiments with similar results and shown as mean values ± S.D. (*) (**) Statistically significant differences ($p < 0{,}05$) and ($p < 0{,}01$) respectively, compared to the control group. (C–D) Spleen cells (1×10^6) were grown in medium alone or activated either with Con A (C) or LPS (D) for 48 h in duplicate cultures and subsequently analysed both by flow cytometry (left panels) and trypan blue exclusion (right panels). The results are presented as total number of Annexin V^+ $7AAD^-$ or 7AAD positive T or B cells in the cultures and represent the mean values of duplicates from one representative experiment out of two performed. The number of viable and dead cells was counted by trypan blue exclusion. These data are presented as the mean values ± S.D. of duplicate cultures from which, three independent cell counts were performed. One representative experiment out of two is shown.

together, the results obtained from these analyses supported the above view that C-Med 100® did not induce a specific block, but rather retarded the progression through various stages of the cell cycle.

3.3. C-Med 100® inhibits proliferation without inducing apoptosis

We next asked whether C-Med 100® would induce apoptotic cell death in treated cells. We addressed this possibility by staining in vitro cultured primary cells and cell lines with the vital dye 7AAD and Annexin V to detect cell death and apoptosis. Thus, Raji cells were cultured as above in the presence or absence of C-Med 100® and were stained and analysed by flow cytometry at various time-points after start of culture. C-Med 100® at 1 mg/ml induced only modest necrotic cell death in Raji cells after 48 h of incubation (Fig. 4A). Most importantly, C-Med 100® rather protected the Raji cells from apoptosis under these conditions. Similar results were obtained at 24 h of incubation (data not shown). Extending the incubation for another 2 days revealed an increased fraction of apoptotic cells in the control cultures while there were more 7AAD positive cells in the C-Med 100® cultures but no increase in apoptosis (data not shown). These results were well in accordance with the observation that C-Med 100® induced inhibition of cell growth but only moderately influenced cell viability as measured by trypan blue exclusion (Fig. 1B).

We also investigated whether C-Med 100® would induce apoptosis in resting and mitogen-activated spleen cells. In unstimulated cultures, C-Med 100® treatment significantly reduced the fraction of Annexin V positive cells as compared to the control cultures (Fig. 4B). Analysis after 72 h of incubation gave similar results (data not shown). Importantly, C-Med 100® did not induce significant cell death, again demonstrating that the extract was only moderately cytotoxic at concentrations inhibiting proliferation.

In cultures stimulated with Con A or LPS for 48 h in the presence of C-Med 100®, there was a dose-

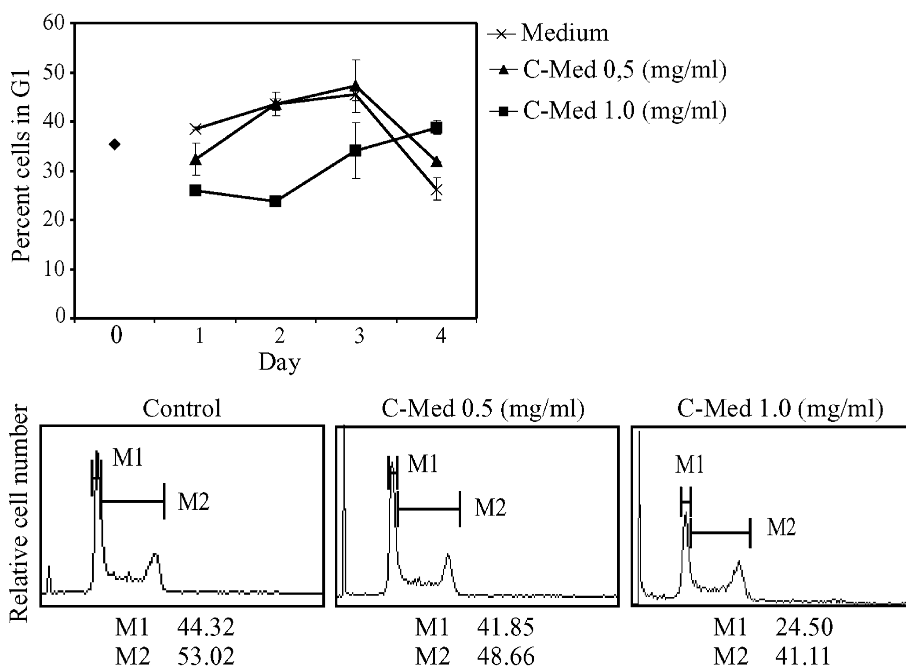

Fig. 5. C-Med 100® does not block cell cycle progression. 2×10^5 Raji cells were cultured in duplicate in the absence or presence of indicated concentrations of C-Med 100®. After indicated time of culture, the cells were stained with propidium iodide and cell cycle analysis performed by flow cytometry. The results in the top panel show mean percent Raji cells in G1 phase ± S.D. and are from one experiment out of two performed. The bottom panels show histograms of cell cycle analysis after 48 h of incubation, from the same experiment.

dependent decrease in number of Annexin V positive T and B cells (Fig. 4C and D, left panels). As detected by 7AAD-staining, there was no increase in numbers of dead cells (7AAD^{+}) in C-Med 100®-treated mitogen-stimulated cultures either. We also performed viable counts of cells from the same cultures. As expected from the proliferation data (Fig. 2A), the number of viable cells decreased dose dependently in the C-Med 100®-treated cultures. However, the number of dead cells in treated cultures was similar to the controls (Fig. 4C and D, right panels). We detected more dead cells using trypan blue exclusion as compared to 7AAD staining, which is probably due to differential sensitivity of the two techniques. These data demonstrate that C-Med 100® mediated inhibition of Con A, anti-CD3 or LPS-induced proliferation was neither due to toxicity nor to induction of apoptosis. In fact, the extract rather protected primary lymphocytes from apoptosis induction.

3.4. C-Med 100® retards cell cycle progression in Raji cells

We next asked whether the observed inhibition of cell growth might be due to a specific block in cell cycle progression. To explore this possibility, Raji cells were incubated for various periods of time in the presence of concentrations of C-Med 100® that significantly inhibited cell growth (Fig. 1A). The cells were then stained with propidium iodide before performing cell cycle analysis by flow cytometry. Our results revealed no specific block in cell cycle progression in cells grown in the presence of C-Med 100® (Fig. 5). Instead, the cells appeared to only progress more slowly through the various stages of the cell cycle compared to Raji cells in control cultures. This is highlighted by the observation that during the first 2 days of culture at 1.0 mg/ml C-Med 100®, a reduced constant fraction of the cells were in the G1-phase and that fraction only subsequently increased to control levels.

3.5. C-Med 100® interferes with NF-κB activation

Since C-Med 100® inhibited proliferation of both mitogen-activated primary lymphocytes and malignant cells, we reasoned that this extract most probably interfered with some major signal-transduction path-

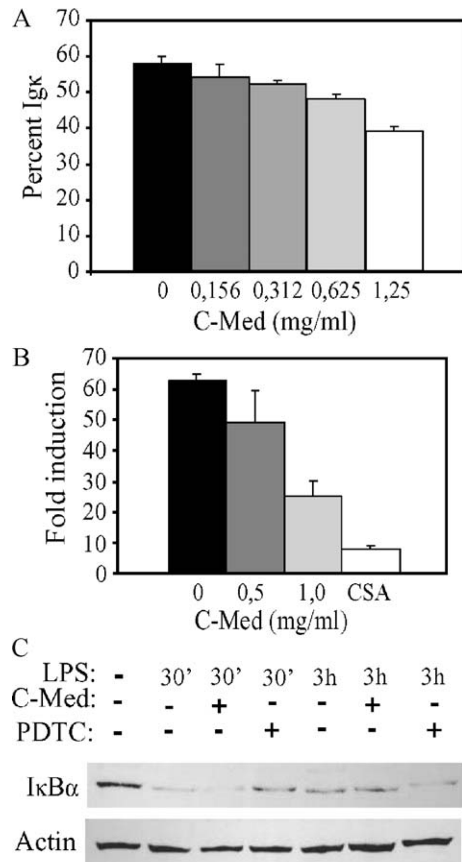

Fig. 6. (A) C-Med 100® inhibits NF-κB activity. 70Z/3 cells (2×10^5) were pretreated for 4 h with indicated concentrations of C-Med 100® before activation for 20 h with LPS (25 μg/ml). The cells were thereafter stained with 7AAD and Igκ-antibodies and analysed by flow cytometry. The depicted data are mean Igκ-positive cells ± S.D. of duplicate cultures from one representative experiment out of three performed. (B) Jurkat T cells transfected with a NF-κB reporter construct were pre-cultured with various concentrations of C-Med 100® for 2 h, PMA and ionomycin were thereafter added and the cells incubated for another 6 h. The cells were then lysed and extracts analysed for luciferase activity. Cyclosporin A (CsA) (1 μg/ml) was used as a positive control for NF-κB inhibition. The presented data are the mean induction of luciferase activity in triplicate cultures from one representative experiment out of four. (C) 70Z/3 cells (5×10^6) were pretreated with either C-Med 100® (1 mg/ml) or with PDTC (100 μM), as a positive control, for 2 h and thereafter stimulated with LPS (25 μg/ml) for indicated time points. Cytoplasmic extracts were thereafter prepared and equal amounts of protein analysed by Western blotting using IκBα-specific antibodies. One representative experiment out of five is presented.

way controlling cell growth and/or division. Similar extracts from *U. tomentosa* were previously shown to inhibit TNFα-production in macrophages [14], suggesting a possible interference with the versatile transcriptional activator NF-κB. It is also well established that the NF-κB protein is involved at several steps in cell cycle progression, further supporting this possibility (reviewed in Refs. [17,18]).

To elucidate whether C-Med 100® might interfere with NF-κB activity, we studied both Igκ expression in 70Z/3 cells [31,32] and the expression of a NF-κB-activated reporter gene in Jurkat cells. The 70Z/3 cells were pretreated with C-Med 100® for 3 h and then stimulated with LPS for 24 h before Igκ-expression was measured with flow cytometry. The results showed that C-Med 100® inhibited the Igκ-expression in a dose-dependent manner (Fig. 6A). Further, we observed that the viability of LPS-treated 70Z/3 cells increased with increasing concentrations of C-Med 100® (data not shown). We confirmed these results using Jurkat T cells transfected with a construct carrying NF-κB activated luciferase reporter gene. The transfected cells were pretreated with C-Med 100® for 2 h and then stimulated with PMA and ionomycin for 6 h before harvest and measurement of luciferase activity. C-Med 100® treatment reduced NF-κB activity with almost 50% (Fig. 6B) and the effect was seen in approximately the same dose interval as for growth inhibition.

To analyse at what level C-Med 100® might interfere with the NF-κB signalling pathway, we investigated the possible effects of this extract on LPS-induced degradation of IκBα in 70Z/3 cells. As can be seen, C-Med 100® did not detectably inhibit the degradation of IκBα (Fig. 6C). Taken together, our results showed that C-Med 100® inhibits the activation of NF-κB in vitro. However, the exact mechanism of this inhibition remains to be established.

4. Discussion

A previous report demonstrated that C-Med 100® inhibits proliferation of human tumor cells by the induction of apoptosis [24]. Using an independent measure of apoptosis and necrosis (i.e. Annexin V/7AAD staining), this study has confirmed and extended the data reported on in the aforementioned previous study. First of all, the C-Med 100® extract used in the present study contained maltodextrin as a spray-drying carrier where as the previous study did not, in turn explaining why fourfold higher doses were required to inhibit proliferation. However, more importantly when we used carrier depleted C-Med 100® in the present study, we obtained similar proliferation inhibition and apoptosis data, suggesting that the carrier does not interfere in the assays and confirmed the data in the earlier study (data not shown). Secondly, the previous and present studies both showed tumor cell lines to be differentially sensitive to apoptosis induction, the HL60 cell line being particularly sensitive. Thirdly, in neither the present or previous studies did C-Med 100® induce significant necrotic cell death. Thus, for tumor cell lines the general conclusion seems to be that C-Med 100® inhibits cell proliferation without inducing cell death at doses that are active in vivo.

C-Med 100® also efficiently inhibited proliferation of primary lymphocytes stimulated with either T or B cell mitogens. The inhibition was more prominent in cultures stimulated with T cell mitogens as compared with the B cell mitogen LPS where higher concentrations of C-Med 100® were required to reach the same level of inhibition. Consistent with the data obtained using tumor cell lines, C-Med 100® neither induced apoptosis in unstimulated nor in mitogen-stimulated primary lymphocytes. On the contrary, we observed a significantly lower fraction of Annexin V positive cells in unstimulated cells, indicating that C-Med 100® rather protected from induction of apoptosis. High C-Med 100® concentrations were cytotoxic and induced cell death both in tumor cells and in primary lymphocytes (data not shown). However, at lower concentrations significant C-Med 100® mediated inhibition of cell proliferation was detectable also in cultures where no cell death was induced. We conclude therefore that induction of proliferation arrest and cell death is independent events. Further, our data suggest that induction of apoptosis by C-Med 100® may be specific to certain tumor cells only.

To understand the basis of proliferation inhibition, we performed cell cycle analysis of treated cells. We did not detect a distinct cell cycle block neither in Raji cells nor in cultures of primary Con A stimulated CD4 or CD8 T cells. In the latter cultures, a smaller fraction of the C-Med 100®-treated cells initiated each consecutive mitosis cycle as compared to control cells, but that observation is also consistent with slower cell cycle

progression. Analysis of LPS-stimulated B cells also supported that view. We suggest therefore that C-Med 100®-treated cells, depending on the dose of extract, progress more slowly through or can be arrested at several stages of the cell cycle. This effect combined with the protection from apoptosis seems unique, since inhibition of cell proliferation usually is mediated through a cell cycle block or an induction of cell death. The possibility that different constituents of the extract induce these effects is currently under investigation.

Previous studies have reported that C-Med 100® enhances repair of both radiation [22] and Doxorubicin induced DNA damage in vivo [23]. Similar enhancement was also observed in humans' [25]. We previously showed that treatment with C-Med 100® prolonged the life span of lymphocytes in vivo [27]. The enhancement of DNA-repair would be expected to protect cells from apoptosis, as observed in here in resting lymphocytes cultured in vitro. Enhanced DNA repair and protection from apoptosis would in turn both be expected to prolong lymphocyte half-life and may thereby increase the number of lymphocytes in vivo. Thus, even though C-Med 100® inhibited mitogen-induced lymphocyte proliferation in vitro, the majority of lymphocytes in a normal mouse is resting and could well accumulate because of prolonged half-life. Because oxygen radicals can both activate NF-kB (reviewed in Ref. [33]) and inhibit DNA repair [34], then the reported antioxidant properties [9,14] of water extracts of *Uncaria* species such as C-Med 100®, may explain the mechanistic interactions leading to increased lymphocyte survival.

The NF-κB pathway appeared to be a possible target of action for C-Med 100®. Indeed, NF-κB activity in 70Z/3 cells stimulated with LPS and expression of a NF-κB driven reporter gene were inhibited by the extract and in the same dose range that caused inhibition of cell proliferation. Our results confirm those in a previous report [15], which showed that another extract from *U. tomentosa* also inhibited NF-κB activation. In addition, that extract did not decrease the viability of HT29 cells or Raw 264.7 cells, but instead significantly reduced chemically induced apoptosis in these cell lines. Taken together, our present results are well in accordance with those data.

The NF-κB pathway has been implicated both in the control of apoptosis induction (reviewed in Ref. [21]) and of cell cycle progression (reviewed in Refs. [17,18]). NF-κB activation is in several ways involved in protecting cells from apoptosis and in particular by controlling the expression of anti-apoptotic genes (reviewed in Refs. [35,36]). Thus, deprivation of NF-κB activity has been shown to promote apoptosis induction which might explain why C-Med 100® treatment induced apoptosis in certain tumor cells [24]. Despite the fact that C-Med 100® reduced NF-κB activity, we failed to detect induction of apoptosis by C-Med 100® treatment in here. The reason for this difference is at present unknown. Importantly, NF-κB is induced during G_0/G_1 transition and inhibition of NF-κB activity has also been shown to retard cell cycle progression in various cell types (reviewed in Ref. [18]). C-Med 100®, which reduces NF-κB activity, would therefore be expected to retard cell cycle progression and inhibit proliferation.

We further analysed the NF-κB signalling pathway and report that there was no inhibition in IκBα degradation in the presence of C-Med 100®. NF-κB activity is, however, regulated at several different levels and other mechanisms such as phosphorylation of NF-κB subunits or regulation of nuclear translocation at the level of the IκBβ-protein might be involved. In conclusion, we observed that C-Med 100® inhibited the activity of a transcription factor that is implicated in the inflammatory responses. This suggests that the reported anti-inflammatory effects from extracts of *U. tomentosa* might be caused through inhibition of this mediator in vivo. Further studies are needed to clarify the exact mechanism of NF-κB inhibition by extracts of *U. tomentosa*. Identification of the active components in the C-Med 100® extract will certainly help in dissecting these mechanisms and also aid in identifying other putative targets of this extract.

Acknowledgements

We thank Eva Miller for expert technical assistance. This work was supported by grants from CampaMed, the Swedish Cancer Society and the Swedish Research Council.

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