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Quinic acid is a biologically active component of the *Uncaria tomentosa* extract C-Med 100®

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Abstract

We have previously reported that the C-Med 100® extract of the plant *Uncaria tomentosa* induces prolonged lymphocyte half life and hence increased spleen cell number in mice receiving the extract in their drinking water. Further, the extract induces cell proliferation arrest and inhibits activation of the transcriptional regulator nuclear factor κB (NF-κB) in vitro. We now report that mice exposed to quinic acid (QA), a component of this extract, had significantly increased number of spleen cells, thus recapitulating the in vivo biological effect of C-Med 100® exposure. Commercially supplied QA (H^+ form) did not, however, inhibit cell proliferation in vitro, while the ammonia-treated QA (QAA) was a potent inhibitor. Both QA and QAA inhibited NF-κB activity in exposed cells at similar concentrations. Thus, our present data identify QA as a candidate component for both in vivo and in vitro biological effects of the C-Med 100® extract.

Keywords: *Uncaria tomentosa*; Inhibition; Proliferation; NF-κB; Cell survival

1. Introduction

Hot water extracts of the vine *Uncaria tomentosa* have been shown to affect the function of the immune system in various ways [1–4]. In particular, such extracts have been found to have various anti-inflammatory effects such as inhibition of the production of the inflammatory cytokine TNFα [5] and the activation of the central transcription factor nuclear factor κB (NF-κB) [6–8]. This factor regulates the expression of proinflammatory cytokines (reviewed in Ref. [9]) and is involved in the regulation of apoptosis (reviewed in Ref. [10]).

C-Med 100® is a hot water extract from the bark of *U. tomentosa*, that has been size-separated to remove molecules larger than 10 kDa such as tannins and flavanoids [11,12]. A number of experimental observations support that components of this extract influence cell survival. Thus, it has been shown to enhance DNA repair [11,13] and to protect both primary lymphocytes and tumor cells from "spontaneous" apoptosis induction in vitro [8].

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Sheng et al. reported that the extract significantly accelerated the recovery of white blood cells (WBC) in rats treated with the chemotherapeutic agent doxorubicin (DXR) [12]. Supplementation with C-Med 100® in humans immunized with pneumococcal vaccine caused a prolonged immune response to the vaccine [14]. Further, there was an elevated number of WBC in both rats and humans exposed to the extract [11]. We have recently made similar observations in the mouse and demonstrated that the number of cells returned to the normal level within a month after withdrawal of the extract [15]. Most importantly, we demonstrated that the increase in cell numbers correlated with prolonged lymphocyte survival, providing a possible explanation to the above experimental observations [15].

Recently, we have shown that the C-Med 100® extract inhibits the proliferation of both tumor cells and primary lymphocytes without induction of apoptosis or necrotic cell death, thereby disconnecting proliferation inhibition functionally from induction of cell death [8]. Furthermore, we reported that this extract also inhibited the activity of the transcription factor NF-κB, an effect previously described for extracts of *U. tomentosa* [6,7].

Our goal was to identify components of the C-Med 100® extract that caused the abovementioned biological responses. Water extracts of *U. tomentosa*, such as C-Med 100®, have been shown to contain 8–10% carboxy-alkyl-esters (CAEs). These are primarily involved in the anti-inflammatory effects associated with the efficacious properties of these extracts (Ref. [16] and Sheng et al. [17] Active ingredient of Cat's Claw water extracts. Identification and efficacy of quinic acid, submitted for publication). In the present report, we identify quinic acid (QA) as a biologically active component of the Cat's Claw extract. We demonstrate that QA increases splenic leukocyte numbers in vivo and inhibits NF-κB activity in cells grown in tissue culture in vitro.

2. Materials and methods

2.1. Mice

C57BL/6 females were bought from M&B, Ry, Denmark and were 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. Extracts and compounds

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) and recently acquired by Optigenex (New York, NY, USA). The extract is water-soluble and ultra-filtered to remove high molecular weight (MW) conjugates (>10 kDa). The extract contains carboxy-alkyl-esters (CAE) as active ingredients (8–10%) and is almost free of oxindole alkaloids (≤0.05%) [11]. The active CAE components have been identified as benzoic acid analogues such as quinic acid [16]. The extract contains no detectable gram negative bacteria and endotoxin. Quinic acid (1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid) was bought from Sigma-Aldrich (Stockholm, Sweden). Both C-Med 100® and QA were dissolved in RPMI medium 30 min before use in in vitro cultures. QA was isolated from C-Med 100®, as already described in detail elsewhere (Ref. [16] and Sheng et al. [17] An active ingredient of Cat's Claw water extracts. Identification and efficacy of quinic acid, submitted for publication). Briefly, C-Med 100® was subjected to TLC chromatography in 1% ammonia in ethanol, eluted from the TLC plates in 1% ammonia in water and crystallized from methanol after neutralization and acidification with HCl.

2.3. In vivo treatment

Mice were fed with C-Med 100® and QA (Sigma-Aldrich), dissolved in autoclaved tap water at indicated concentrations for 21 days. The drinking water bottles were changed every third day. The animals were then sacrificed, the spleens removed and blood samples collected. The blood samples were analyzed in a Sysmex KX-21N cytometer (Sysmex, Kobe, Japan).

2.4. Fluorochrome-conjugated reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-CD8α (YTS169.4), anti-CD4 (GK1.5), anti-B220

(RA3.6B2), anti-Igκ and Cyanin 5 (Cy-5)-conjugated anti-B220 (RA3.6B2) were prepared in our laboratory. Phycoerythrine (PE)-conjugated anti-CD4 (RM4-5) and anti-CD8α (53-6.7) and allophycocyanin (APC)-conjugated TCRβ (H57-597) were bought from BD Biosciences (San Diego, CA). 7-amino-actinomycin D (7AAD) was bought from Sigma-Aldrich. Annexin V-FITC was bought from Molecular Probes (Leiden, Holland).

2.5. 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 [15]. Spleen cells were preincubated for 10 min on ice with the anti-Fc-receptor antibody 2.4G2 (FcγRIII/II; prepared in our laboratory) to prevent nonspecific binding to Fc-receptors. The cells were analysed with a FACSCalibur flow cytometer (Becton Dickinson, San José, CA).

2.6. Cell cultures

The Raji human Burkitt's lymphoma (CCL-86), the Jurkat human acute T cell leukaemia (TIB-152), 70Z/3 mouse pre-B lymphocyte cell line (TIB-158) 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-mercaptoethanol and 1 mM sodium pyruvate (all supplements from Life Technologies) at 37 °C, 5% CO_2. The number of Raji cells in duplicate cultures was determined with a Coulter Z1 cell counter (Beckman Coulter, Fullerton, CA, USA) three times each.

The number of viable and dead cells was determined using trypan blue exclusion by counting three independent samples from duplicate cultures. Cells were stained with 2 μg/ml 7-amino-actinomycin D (7AAD; Sigma-Aldrich, St Louis, MO, USA) and with Annexin V (Molecular Probes), according to the manufactures' protocol, and the cells were defined as apoptotic (Annexin V^+ $7AAD^-$) or dead ($7AAD^+$) by flow cytometry. Spleen cells were polyclonally activated with 2.5 μg/ml concanavalin A (Con A; Amersham Pharmacia, Uppsala, Sweden) or 10 μg/ml lipopolysaccharide (LPS; Sigma-Aldrich). PMA (50 ng/ml) and ionomycin (1 μM; both from Sigma-Aldrich) and pyrollidine dithiocarbamate (PDTC; 100 μM, EMD Bioscience, Calbiochem, San Diego, CA, USA) were used in some cultures as indicated. Proliferation was detected by measuring thymidine incorporation after a 4-h pulse with 1 μCi ^{3}[H]-thymidine (Amersham Pharmacia).

2.7. Transient transfection and analysis of luciferase activity

The reporter construct containing NF-κB binding sequences and the luciferase reporter gene was previously described [18]. Jurkat T cells were transiently transfected with the construct, using the lipofectin method, as described by the manufacturer (Life Technologies). After transfection, the cells were rested for 22 h, pooled and precultured for 2 h in the presence or absence of C-Med 100® or QA before stimulation. After 6 h of stimulation, the cells were harvested and washed twice in phosphate-buffered saline (PBS). The cells were lysed, and aliquots of the lysates were analyzed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI). Luminiscence was quantitated in a MicroLumat LB 96 P luminometer (EG and G Berthold, Wallac Sverige, Upplands Väsby, Sweden).

2.8. Preparation of cell extracts

The 70Z/3 cells were pretreated with QA (1 or 2 mg/ml) or with PDTC for 2 h before they were stimulated with LPS for various time points. Whole cell extracts from the 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 MgCl2, 2% NP-40, 1 mM PMSF and a protease inhibitor cocktail; Roche Diagnostics Scandinavia) and incubated on ice for 10 min. The cell debris was pelleted, and the supernatants were stored at −70 °C until Western Blot was performed.

2.9. Western blotting

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, the membrane was incubated for 2 h with primary antibodies specific either to IκBα or to phosphorylated IκBα (both from Cell Signaling Technology, Beverly, MA). The membranes were thereafter washed three times in TBST and incubated with HRP-conjugated goat antirabbit antibodies (Amersham Pharmacia). The membranes were washed three times and chemoluminescence was detected using the ECL-reagent (Amersham Pharmacia) and X-ray film (CEA, Strängnäs, Sweden).

2.10. Statistics

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

3. Results

3.1. A biologically active component of C-Med 100®

A biologically active component of C-Med 100® was isolated, as described in Materials and methods. The eluted material from one fraction was found to inhibit proliferation of HL-60 cells similarly to the C-Med 100® extract itself. A component in this biologically active fraction was identified by MALDI mass spectrometry as quinic acid (QA), as described in detail elsewhere (Sheng et al. [17] An active ingredient of Cat's Claw water extracts. Identification and efficacy of quinic acid, submitted for publication). We therefore attempted to confirm this as well as other known biological activities of the C-Med 100® extract, using commercially available QA.

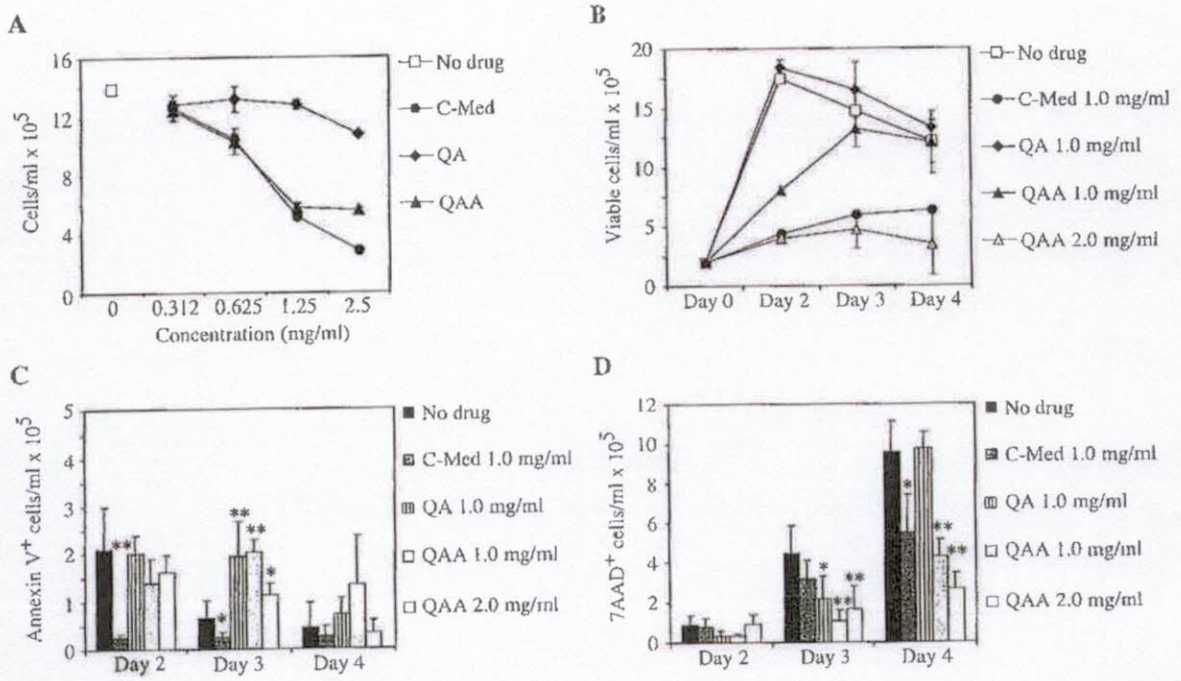

Fig. 1. QAA, but not QA, inhibits Raji cell proliferation. (A) Raji cells (2×10^5) were cultured in the presence of indicated concentrations of C-Med 100®, QAA or QA. Growth rate was assayed after 48 h by counting triplicate samples of duplicate cultures in a Coulter cell counter. Results from one representative experiment out of two performed are presented as mean values of cell/ml±S.D. (B) Triplicate samples of cells from duplicate parallel cultures were stained with trypan blue at indicated times and counted. The data show the mean number of viable cells/ml±S.D. from one out of two similar experiments. (C and D) C-Med 100®, QAA and QA do not induce cell death. Cells from the same cultures as in panel (B) were stained with Annexin V and 7AAD and analyzed by flow cytometry. The results are presented as the mean number of Annexin V^+ $7AAD^-$ (apoptotic) or $7AAD^+$ (dead) cells±S.D. of duplicates from three experiments with similar results. Statistically significant differences (*$p<0.05$) and (**$p<0.01$), compared to Raji cells grown in medium (no drug).

However, commercially available QA neither inhibited the proliferation of Raji tumor cells (Fig. 1A,B) nor proliferation of ex vivo murine lymphocytes (Fig. 2A). One possible explanation for the functional discrepancy between commercial QA and the QA isolated from C-Med 100® might relate to the fact that QA isolated from C-Med 100® was treated with ammonia during chromatography and elution on silica gel. To test this possibility, we treated commercially available QA with 1% ammonia, under identical conditions to those used to isolate QA from C-Med 100® using TLC, and then analyzed its biological activity compared to commercial QA. Consistent with our hypothesis, ammonia-treated QA (denoted QAA)

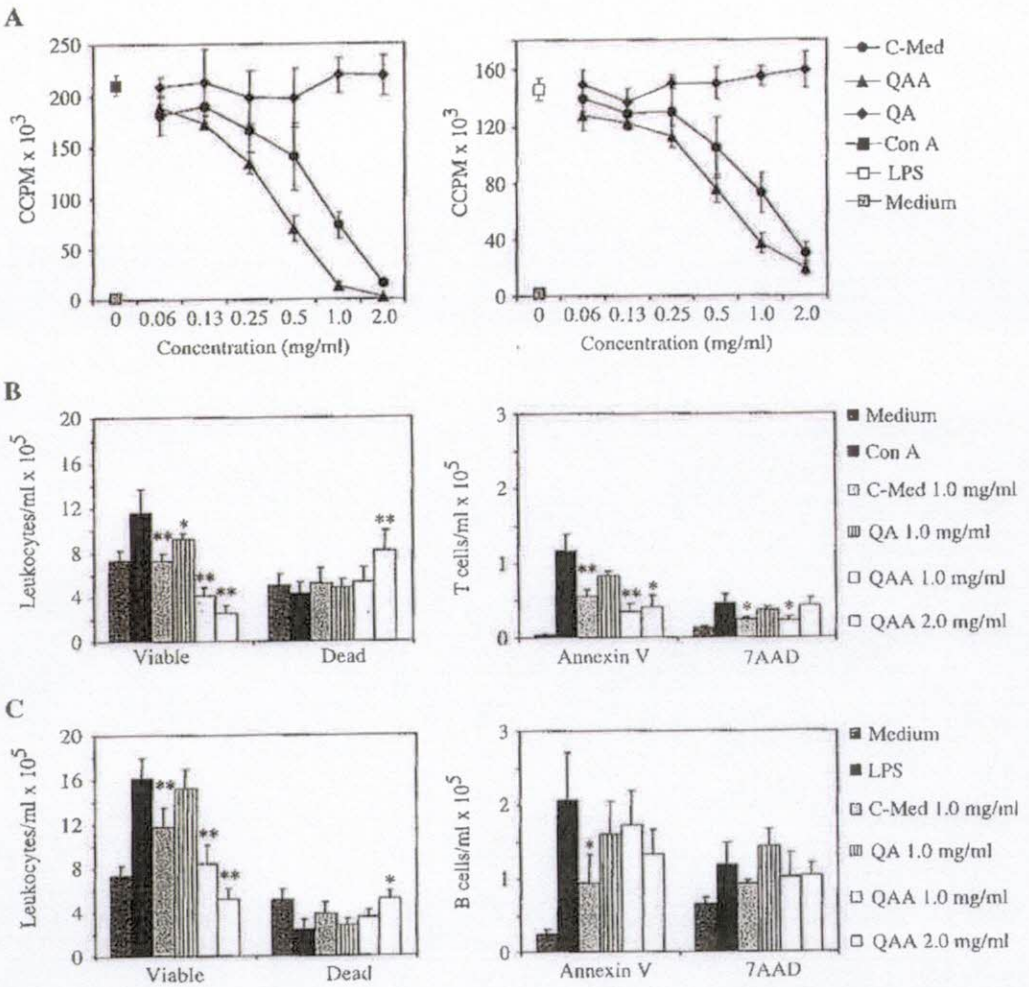

Fig. 2. QAA, but not QA, inhibits proliferation of mitogen-stimulated mouse lymphocytes. (A) Spleen cells (2×10^5) were activated with Con A (left; 2.5 μg/ml) or LPS (right; 10 μg/ml) in the presence of indicated concentrations of C-Med 100®, QAA or QA. Proliferation was assayed after 48 h by ^{3}[H]-thymidine incorporation in triplicate cultures. Results from one representative experiment out of three are presented as mean values±S.D. (B and C; left) Neither C-Med 100®, QAA nor QA induce cell death. Cells from parallel cultures were stained with trypan blue, and the mean absolute number of viable and dead cells/ml from one out of three similar experiments are shown. (B and C; right) C-Med 100®, QAA and QA do not induce apoptosis. Aliquots of the above cultures were stained with Annexin V and 7AAD and analyzed by flow cytometry. The results are presented as the mean number of Annexin V^+7AAD^- (apoptotic) and $7AAD^+$ (dead) cells/ml±S.D. of duplicates from three experiments with similar results. Statistically significant differences (*$p<0.05$) and (**$p<0.01$) compared to the control cultures stimulated with Con A (B) or LPS (C) alone.

inhibited cell proliferation in a dose-dependent manner (Figs. 1A,B and 2A) without being overtly toxic (Figs. 1D, 2B and 2C). As previously reported [8] and confirmed here, C-Med 100® consistently reduced the fraction of apoptotic cells both in cultures of tumor cells (Fig. 1C) and normal lymphocytes (Fig. 2B,C). QAA also significantly reduced the fraction of apoptotic T cells, while there was a tendency to reduction (p=0.053) in parallel cultures treated with QA (Fig. 2B). This effect was not seen in Raji cells (Fig. 1C) nor in normal B cells (Fig. 2C), 70Z/3 cells or Jurkat T cells (data not shown). We have not further investigated this discrepancy, and it remains a focus of our future research.

3.2. QA inhibits NF-κB activity

Our previous study [8] as well as reports from other laboratories [7,9] have indicated that extracts of *U. tomentosa* inhibit NF-κB activity in cells cultured in vitro. To ask whether QA might inhibit this transcriptional regulator, we used Jurkat T cells transfected with a NF-κB-dependent reporter gene. The results in Fig. 3A (left) demonstrate that QA, in a dose-dependent manner, inhibited the NF-κB activity induced by activating the Jurkat T cells with PMA and ionomycin. The inhibition was observed at concentrations of QA that did not induce cell death (Fig. 3A, right). As would be expected from the data presented above, parallel experiments confirmed that QA did not inhibit proliferation of the Jurkat T cells either (data not shown). Thus, QA inhibits NF-κB activity without inhibiting proliferation neither of normal cells nor of tumor cells. QAA inhibited the NF-κB activity to a similar extent as QA (data not shown).

The LPS-induced Igκ-chain expression in 70Z/3 cells is NF-κB-dependent [19]. QA and C-Med 100® inhibited Igκ-chain expression to a similar extent (Fig. 3B, left) without causing cell death (Fig. 3B, right). We used this model to look further into the mechanism of NF-κB inhibition. As can be seen, QA inhibited the LPS-induced breakdown of IκBα in these cells (Fig. 3C), suggesting a plausible mechanism for the inhibition of NF-κB-dependent reporter gene transcription (Fig. 3A). The antioxidant pyrollidine dithiocarbamate (PDTC) as expected [20] inhibited both the breakdown of IκBα and its resynthesis. However, QA did not affect the phosphorylation of IκBα, which in contrast was potently inhibited by PDTC (Fig. 3D). The QA-induced inhibition of IκBα breakdown is thus controlled at another level than IκBα phosphorylation. We have not further investigated the exact level at which QA inhibits the NF-κB activity.

3.3. Increased spleen cell number in QA-treated animals

Our previous report showed that in vivo treatment of mice for three weeks with the C-Med 100® extract increased the number of spleen cells, due to the prolongation of lymphocyte half life [15]. We confirmed this observation here (Fig. 4A), using the previously determined optimal concentration of C-Med 100® (4 mg/ml in the drinking water). The observation that a higher concentration of the extract (8 mg/ml) did not increase splenic lymphocyte numbers further, together with our previous results, demonstrates that this biological effect is only seen in a narrow concentration range. Mice fed with drinking water containing 4 mg/ml C-Med 100® also had significantly higher spleen weight. However, neither of the groups fed with C-Med 100® had significant changes in body weight.

Inasmuch as QA decreased NF-κB activity in treated cells in vitro, we wondered whether this component might also be involved in the above in vivo biological response. To address this possibility, mice were fed with drinking water containing various concentrations of QA. Mice fed with 2 mg/ml of QA had a significantly increased number of spleen cells, but similarly to what was observed in C-Med 100® treated mice, the increase was seen only in a narrow concentration range (Fig. 4A). We confirmed that the increased spleen cell number was paralleled by significantly increased absolute numbers of the major lymphocyte subsets $CD4^+$ T cells, $CD8^+$ T cells and B cells [15] (Fig. 4B), and importantly, that was also the case in the QA-treated animals. Taken together, these data strongly indicate QA as one candidate compound of this in vivo biological effect of C-Med 100®.

Despite the significant increase in spleen cell number, there was no increase in WBC, blood lymphocytes or red blood cells (RBC) in C-Med

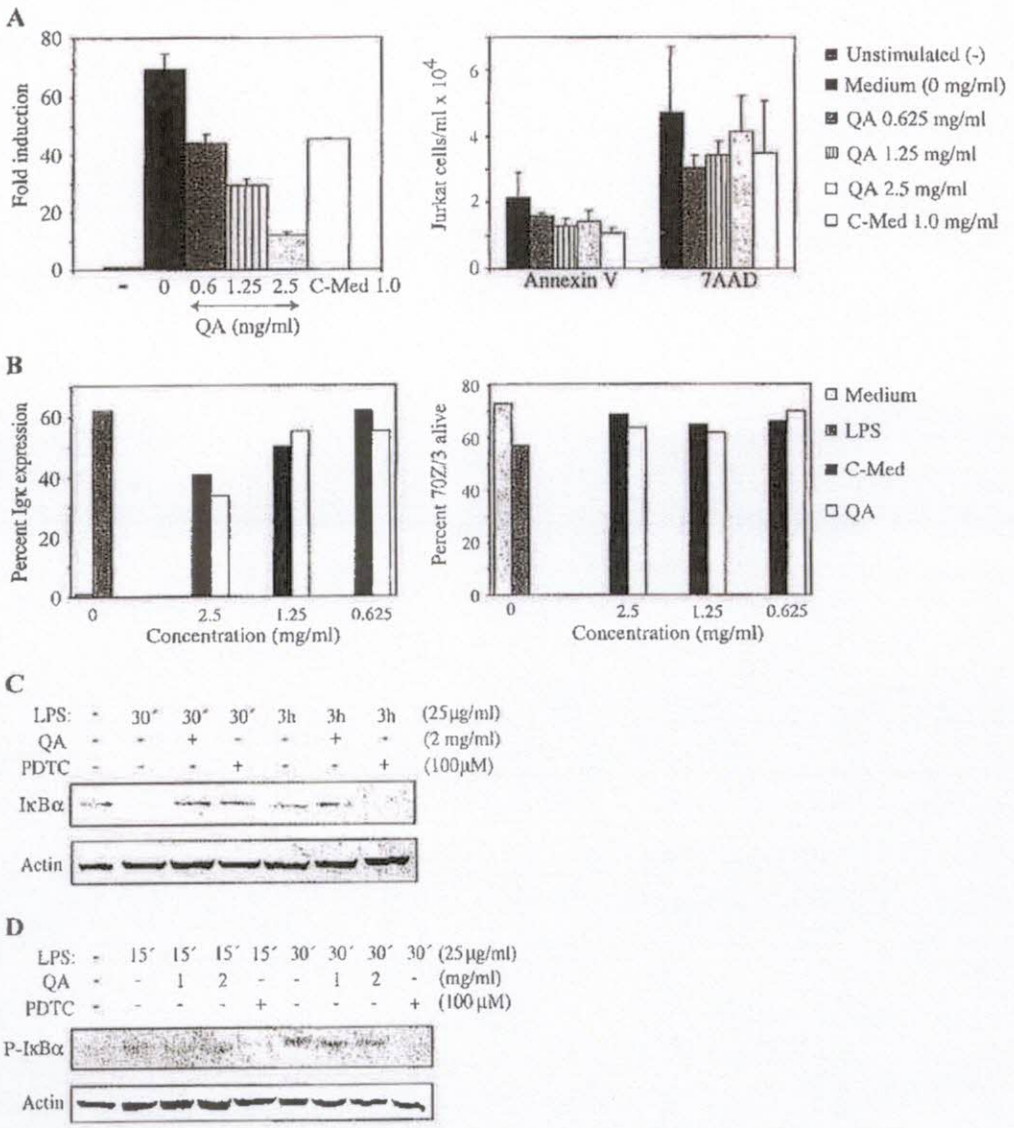

Fig. 3. C-Med 100® and QA inhibit NF-κB activity. (A) Jurkat T cells transfected with a NF-κB reporter construct were precultured with various concentrations of QA or C-Med 100® for 2 h, PMA (50 ng/ml) and ionomycin (1 μM) were thereafter added, and the cells were incubated for another 6 h. The mean induction of luciferase activity in triplicate cultures from one representative experiment out of four are shown (left). QA induced no cell death or apoptosis in Jurkat T cells at concentrations which inhibited NF-κB activity. Jurkat T cells were incubated with various concentrations of QA for 24 h and thereafter stained with Annexin V and 7AAD before analysis by flow cytometry. The data are the mean values±S.D. of triplicates from one representative experiment out of two performed (right). (B) 70Z/3 cells (2×10^5) were pretreated for 4 h with C-Med 100® or QA before activation for 20 h with LPS (25 μg/ml). The cells were thereafter stained with 7AAD and Igκ-antibodies and analyzed by flow cytometry. The results are mean Igκ-positive cells±S.D. (left) and mean 7AAD negative cells±S.D. (right) of duplicate cultures from one representative experiment out of two performed. (C and D) 70Z/3 cells (5×10^6) were pretreated with QA (2 mg/ml) or with PDTC (100 μM) as a positive control for 2 h and thereafter stimulated with LPS (25 μg/ml) for the indicated time. Cytoplasmic extracts equalized for protein concentration were analyzed by western blotting using IκBα-specific antibodies. One representative experiment out of (C) three and (D) two is presented.

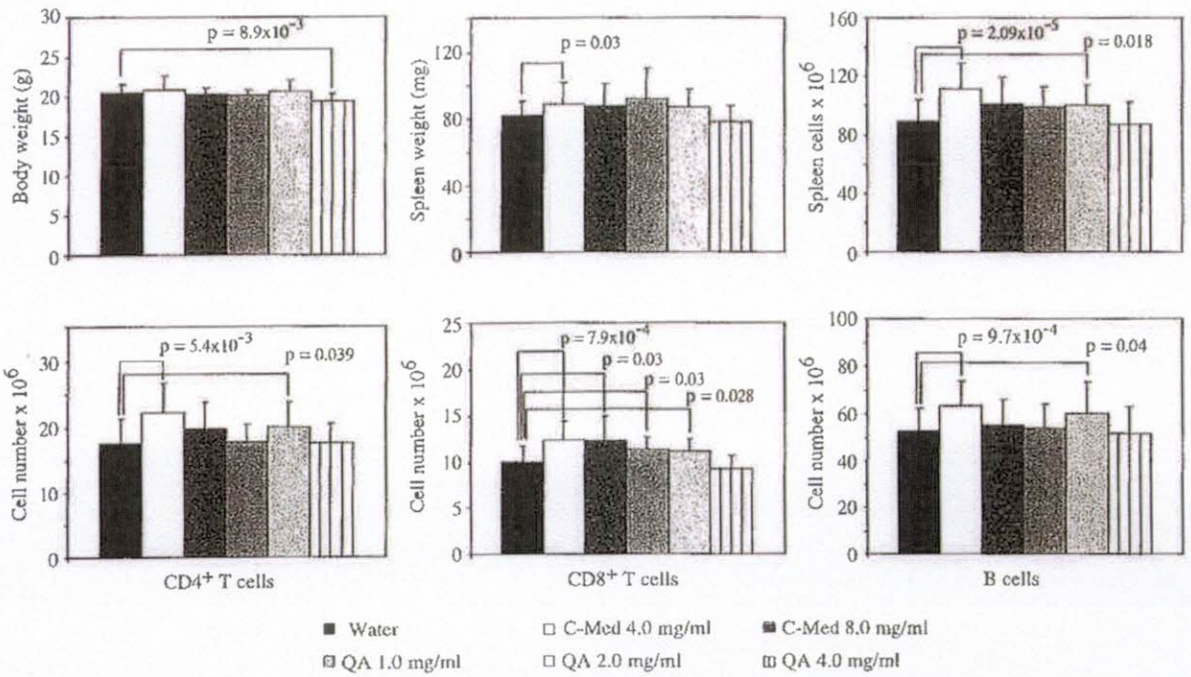

Fig. 4. Increased spleen cell number in QA and C-Med 100®-treated mice. Mice were treated with indicated concentrations of QA or C-Med 100® in the drinking water for 21 days, sacrificed and absolute number of spleen cells counted using trypan blue exclusion. The presented data are mean cell numbers±S.D. pooled from five experiments (water, n=21; C-Med 100® 4 mg/ml, n=24; C-Med 100® 8 mg/ml, n=9; QA 1 mg/ml, n=9; QA 2 mg/ml, n=22; QA 4 mg/ml, n=10). Statistically significant differences compared to control mice supplemented with tap water are indicated.

100®-treated mice (Fig. 5A,B). In contrast, mice treated with increasing concentrations of QA had increasing number of WBC and blood lymphocytes. In the group treated with 4 mg/ml of QA, this increase was significant as compared to normal control animals. However, the body weight of those animals was also significantly reduced, so the significance of this observation is difficult to evaluate.

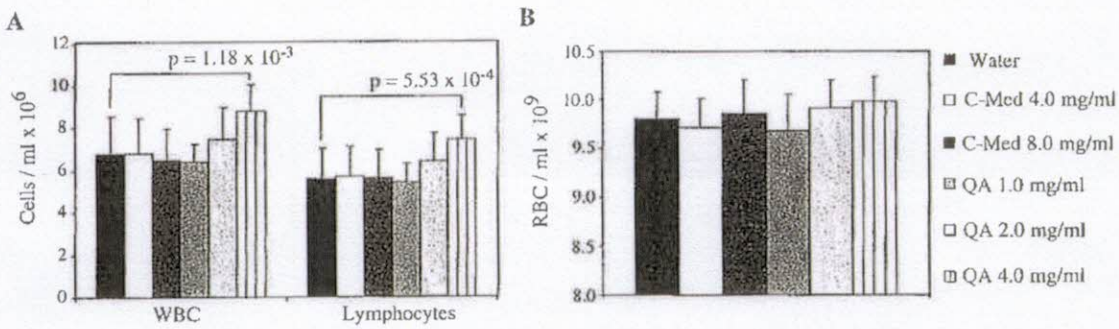

Fig. 5. Increased number of WBC in QA-treated mice. Mice were treated with indicated concentrations of either QA or C-Med 100® in the drinking water for 21 days and thereafter sacrificed. The number of WBC, (A) lymphocytes and (B) erythrocytes (RBC) in peripheral blood was determined using an automatic cell counter. The data are presented as the mean number of cells/ml±S.D. from five experiments (water, n=21; C-Med 100® 4 mg/ml, n=24; C-Med 100® 8 mg/ml, n=9; QA 1 mg/ml, n=9; QA 2 mg/ml, n=22; QA 4 mg/ml, n=10). Statistically significant differences compared to control mice supplemented with tap water are indicated.

4. Discussion

This report identifies QA as one candidate component responsible for some of the biological effects ascribed to the *U. tomentosa* extract C-Med 100® both in vivo and in vitro. This conclusion was based on a number of chemical and biological evaluations. (i) C-Med 100®, precipitated with 90% methanol and chromatographed on TLC plates in ethanol containing 1% ammonia, have only one band (R_f=0.3) containing the desired biological activity. (ii) This particular band on the TLC plate could only be eluted from the silica gel, using 1% ammonia diluted either in alcohol or in water. (iii) When the bioactive fraction was neutralized by acidifying with 1N HCl, only QA acid could be structurally elucidated (Ref. [16] and Sheng et al. [17] An active ingredient of Cat's Claw water extracts. Identification and efficacy of quinic acid, submitted for publication). (iv) Analytical standard QA did not have the biological activity associated to C-Med 100®. However, when subjected to the same 1% ammonia base treatment as C-Med 100® and thereafter lyophilized to dryness, thus keeping it in the ammonia form, it became bioactive. Taken together, these data strongly suggest that QA is an important bioactive component of C-Med 100®, although it probably does not occur naturally in the free acid form. More likely because of the demonstrated C-Med 100® sensitivity to ammonia, a novel salt, chelate or hydrolyzable ester are more favorably indicated rather than a simple ammonium salt that would be 100% dissociated in water to the anionic and cationic forms. We showed before [8] and confirmed here that C-Med 100 potently inhibited the proliferation of both normal and tumor cells. In accordance with this, QAA had similar biological activity on cell proliferation.

In this report, we fed mice with pure QA in the drinking water for 3 weeks and observed, as was previously seen in C-Med 100® fed mice [15], that there was a significant increase in splenic lymphocytes in experimental animals as compared to normal controls. The absolute cell number of all major lymphocyte subsets had increased in the treated mice, suggesting that QA, similarly to the C-Med 100® extract, may prolong lymphocyte half life. Our previous data showed that the increased spleen cell number was fully reversible after interrupting exposure to C-Med 100® and without any detectable pathological changes in the treated animals [15]. Interestingly, the impact of both C-Med 100® and QA on splenic cell numbers was highly dose-dependent (Ref. [15] and Fig. 4) with an optimal dose above which the biological effect was reversed. The reason for this is not known but might be due to saturation of metabolic pathways by the high doses. This might lead both to the accumulation of free QA in serum and potentially to the increased role of the liver in the metabolism, as has been observed in situations of administering high doses of polyphenols [21]. Collectively, our data strongly indicate that QA is one of the components in the C-Med 100® extract, responsible for its capacity to increase spleen cell numbers.

We also monitored the number of WBC and lymphocytes in peripheral blood of the treated animals. While there were no significant changes in C-Med 100®-treated animals, there was a dose-dependent increase in both WBC and blood lymphocytes in QA-treated animals. At the highest dose of QA administered (4 mg/ml), the increase in blood cell number was paralleled by a slight decrease in spleen cell number, suggesting that these two observations might be connected. However, these animals had also lost body weight compared to the controls, indicating that this high a dose also had other effects on the animals.

As previously reported [8] and confirmed here, exposure to C-Med 100® also had a significant antiapoptotic effect on cells at concentrations inhibiting proliferation. However, the number of apoptotic cells was not reduced in cultures exposed to QA or QAA, suggesting that other components of the extract might be responsible for this effect.

Extracts of *U. tomentosa* inhibit the activity of the transcriptional regulator NF-κB [6,7], and this is most probably one of the reasons for the anti-inflammatory properties [5] of such extracts. We have previously shown that the C-Med 100® extract also inhibited NF-κB activity but without inhibiting degradation or expression of IκBα [8]. The data presented here indicate that QA, in a dose-dependent fashion, inhibited the expression of a NF-κB-dependent reporter gene in tissue culture cells. A similar level of inhibition was seen using similar concentrations of QAA (data not shown). However, in contrast to the C-Med 100® extract, QA inhibited the degradation of IκBα. These data collectively suggest that QA and C-

Med 100® inhibited NF-κB activity by different mechanisms. Further, QA exposure did not detectably interfere with the phosphorylation of IκBα while it inhibited the degradation of that protein, suggesting that QA-induced inhibition of NF-κB activity is regulated at another level. It may seem paradoxical that QA, which is a potent inhibitor of LPS-induced NF-κB activity in 70Z/3 cells, does not inhibit LPS-induced proliferation of normal B cells. However, the toll-like receptor 4 (TLR4)-mediated induction of the MAP-kinase pathway (reviewed in Ref. [22]) may still occur in these cells and be sufficient to induce proliferation.

C-Med 100® treatment was previously shown to accelerate the recovery of blood cells after chemically induced leukopenia in the rat [12]. Further, we have shown that in vivo treatment prolongs lymphocyte half life, leading to the accumulation of spleen cells in treated animals. This effect was dependent on the continuous presence of the extract, as lymphocyte numbers regained normal levels within a few weeks of terminating the treatment. As C-Med 100® has a clear antiapoptotic effect on cells grown in vitro, it is tempting to speculate that the accumulation of lymphocytes might be caused by this property of the extract. Further, NF-κB is also known to be involved both in controlling cell division (reviewed in Refs. [23,24]) and cell survival (reviewed in Ref. [25]), therefore suggesting that interference with the expression level of this transcriptional regulator might be involved in this in vivo phenomenon.

In plant extracts, QA can occur as an ester with caffeic acid, forming chlorogenic acid, a major component in coffee [26]. On the other hand, some fruits and berries such as cranberries and sea Buckthorn are particularly rich in free QA [27,28]. The absorption of dietary chlorogenic acid by both human and rodents is well-documented [29–31]. It has been shown that gut microflora play an important role in the absorption of this compound by providing the esterases hydrolyzing chlorogenic acid into its constituent QA and caffeic acid [31,32] components. The QA component could then be subsequently further metabolized in tissues [31].

QA is a metabolite of the shikimate pathway found in plants and is in fact the major synthetic route to most of the known bioactive ingredients of plant extracts (reviewed in Ref. [33]). Therefore, one would expect that QA, which is water soluble, should be found in a hot water plant extract such as C-Med 100®. The implication of our finding here is that other aqueous extracts of plants may contain QA, and therefore, also have similar biological effects as C-Med 100®.

As suggested before (Sheng et al. [17] An active ingredient of Cat's Claw water extracts. Identification and efficacy of quinic acid, submitted for publication), QA esters may be responsible for the "QA-like" activity of the C-Med 100® extract and cause the accumulation of leukocytes seen in vivo in C-Med 100®-treated animals [15]. Thus, although the extract content of free QA is low, the content of "QA-equivalents" in the form of esters, chelates or salts could contribute significantly to that in vivo biological effect. It should be pointed out that although we have shown that QA recapitulates one of the in vivo effects of C-Med 100®, there might also be other components contributing to the biological response. Such components might synergize with QA and could explain why a relatively high concentration of native QA is required to obtain a similar in vivo effect as with C-Med 100® itself.

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