Article

Inhibitory Effects of Berberine on the Activation and Cell Cycle Progression of Human Peripheral Lymphocytes

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The immunosuppressive property of berberine, an isoquinoline alkaloid, has been well documented, but the mechanism of its action on lymphocytes has not been completely elucidated. The present study is to investigate the effect of berberine on the activation and proliferation of lymphocytes, in particular T lymphocytes. Whole peripheral blood from healthy donors was stimulated with phytohemagglutinin (PHA) alone or phorbol dibutyrate (PDB) plus ionomycin, and the expression of CD69 and CD25 on T lymphocytes was evaluated with flow cytometry. The distribution of cell cycles and cell viability were analyzed by staining with propidium iodide (PI) and 7-aminoactinomycin D (7-AAD), respectively. The results showed that 100 μmol/L and 50 μmol/L of berberine significantly inhibited CD69 expression on T cells stimulated with PDB plus ionomycin or PHA, whereas the effect of 25 μmol/L berberine was not significant. As the incubation time increased, the extent of inhibition decreased. Similarly, the expression of CD25 was also reduced by berberine in a dose-dependent manner over the concentration range of 25-100 μmol/L. Besides, this alkaloid could block lymphocyte cell cycle progression from G_0/G_1 phase to S and G_2/M phase without phase specificity. Moreover, analysis following 7-AAD staining revealed that berberine had no significant cytotoxicity on lymphocytes. Taken together, berberine significantly inhibits the expression of activation antigens on T lymphocytes and also blocks the progression of cell cycles of lymphocytes, suggesting that berberine may exert immunosuppressive effect through inhibiting the activation and proliferation of T cells. *Cellular & Molecular Immunology.* **2005;2(4):295-300.**

Key Words: berberine, lymphocyte, activation antigen, cell cycle

Introduction

Berberine (Ber) is an isoquinoline alkaloid extracted from the Chinese herb Huanglian and many other plants such as the *Berberidaceae* family. It possesses a variety of biological and pharmacological activities, including antibiotic activity (1), anti-tumor (2) and anti-platelet aggregation properties (3).

Extracts of berberine-containing plants have been used for many centuries in the treatment of diarrhea in China, probably through inhibition of mucosal chloride secretion (4). Accumulated evidence in the last decade indicates that berberine has anti-inflammatory activity in animal model and other experimental systems. For example, it has been shown that berberine suppresses the delayed-type hypersensitivity (DTH) reaction (5, 6) and alleviates the clinical signs of adjuvant-induced arthritis in rats (6). In an experimental autoimmune tubulointerstitial nephritis model (7), berberine is found to be effective in decreasing the intensity of pathological injuries in mice and has immunosuppressive effect in this model, too. There is accumulated evidence showing that berberine has potential immunomodulatory effect (5-8), although the mechanism of its action on lymphocytes, in particular T lymphocytes, has not been completely elucidated.

In the present study, we examined the effect of berberine on the expression of CD69 and CD25 activation antigens of human peripheral T lymphocytes, and investigated the effect of berberine on cell cycles of activated lymphocytes *in vitro*,

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which should at some extent account for its immuno-modulatory effect.

Materials and Methods

In vitro activation of human peripheral lymphocytes
Heparin anti-coagulated peripheral blood was collected from healthy volunteers aged from 18-35 and diluted at the ratio 1:2 with RPMI-1640 medium supplemented with 10% (v/v) fetal bovine serum, 50 μmol/L mercaptoethanol, 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco, USA). The diluted blood (1 ml/well in 24-well plate) was preincubated with various concentration of berberine (Sigma, St. Louis, MO, USA) ranging from 25 to 100 μmol/L, in a humidified atmosphere with 5% CO_2 at 37°C for 30 min, followed by stimulation by phorbol 12,13-dibutyrate (PDB, 5.0×10^{-8} mol/L; Calbiochem, USA) plus ionomycin (0.5 μg/ml; Sigma, USA), or phytohemagglutinin PHA-P (10 μg/ml; Sigma), respectively.

Fluorescent antibody staining of the lymphocytes
After culture for appropriate time, 120 μl diluted blood was concentrated by centrifugation to 40 μl and the blood samples were stained for 20 min at 4°C with 10 μl anti-hCD3-CyChrome and 10 μl anti-hCD69-APC or anti-hCD25-APC (all from PharMingen, San Diego, CA, USA), respectively. The red blood cells were lysed and the nucleated cells were collected by centrifugation (1,500 rpm, 5 min). The cell pellets were washed twice with PBS (phosphated-buffered saline, pH 7.4) and fixed with 400 μl paraformaldehyde (4%, prepared in PBS) for 20 min. The samples were ready for analysis with flow cytometry. We had noticed that berberine interferes with staining of FITC and PE labeled antibodies and this has great influence on the accuracy of the flow cytometric analysis, therefore CyChrome (excitation at 488 nm) and APC (excitation at 635 nm) labeled antibodies were adopted for all experiments.

Flow cytometric analysis
The samples were analyzed by a flow cytometer FACSCalibur (Becton Dickinson, USA) and the data were collected and analyzed with CellQuest software (Becton Dickinson). CyChrome was excited with laser at 488 nm and APC excited with laser at 635 nm. The same equipment settings were used for all determination and the parameters of 10,000 cells were collected for each sample.

Assessment of cell viability by 7-aminoactinomycin D
Nucleated cells were collected from 120 μl cultured blood. The cells were then stained with 4 μg/ml 7-aminoactino-mycin D (Sigma) at 4˚C for 15 min as described (9). The cell viability was analyzed with FACSCalibur with excitation wavelength at 488 nm and emission at 650 nm. The live cells exclude the dye whereas dead cells are stained.

Flow cytometric analysis of the cell cycle
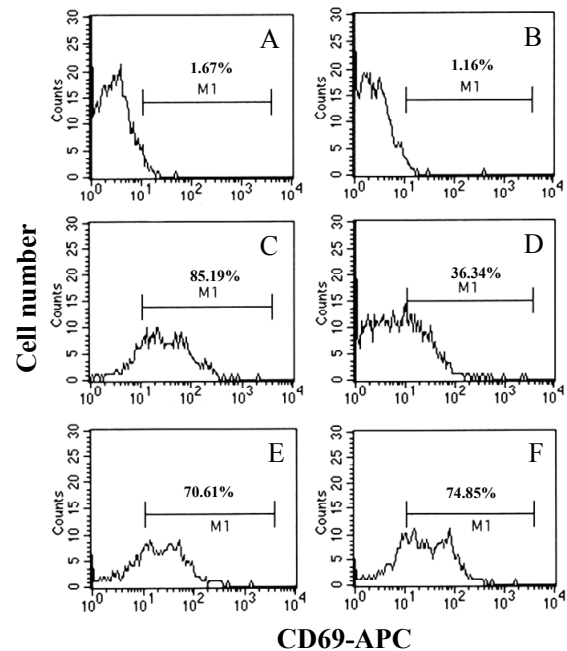The sample preparation was performed as previously

Figure 1. **Flow cytometric analysis of the inhibitory effect of berberine (4 h incubation) on the expression of CD69 on CD3+ lymphocytes.** One representative experiment was shown and figures in each panel are the expression percentages of CD69 on T lymphocytes gated by CD3-CyChrome. Panel A, control; Panel B, Ber (50 μmol/L); Panel C, PDB + Ion; Panel D, PDB + Ion + Ber (100 μmol/L); Panel E, PDB + Ion + Ber (50 μmol/L); Panel F, PDB + Ion + Ber (25 μmol/L). PDB, phorbol 12,13-dibutyrate; Ion, ionomycin; Ber, berberine.

reported (10). Briefly, the diluted blood was incubated for 72 h and nucleated cells were collected. The collected cells were washed once with 2 ml PBS and fixed at 4°C with 1 ml cold 70% ethanol for at least 30 min. Ethanol was removed by centrifugation at 2000 rpm for 5 min and 2 ml PBS was added to wash the pellets. The cellular DNA was stained with propidium iodide (PI) solution (50 mg/ml, 0.1% Triton X-100, 0.1 mmol/L EDTA and 50 mg/ml RNase A in PBS) for 30 min at room temperature and stored at 4°C until measurement. Data of DNA content of the stained cells were acquired on FACSCalibur and analyzed with CellQuest software.

Statistical analysis
Data were summarized as mean ± SD and analyzed by one-way analysis of variance followed by Scheffe's multiple comparison tests. A significance level of 0.05 was used for all comparisons.

Results

Inhibitory effect of berberine on the expression of CD69 on T lymphocytes
The expression of CD69, an early activation antigen for T

Table 1. The inhibitory effect of berberine on the expression of CD69 or CD25 on T lymphocytes stimulated by PDB plus ionomycin

Group	$CD3^+CD69^+/CD3^+$ (%)		$CD3^+CD25^+/CD3^+$ (%)
	4 h	22 h	22 h
Control	1.35 ± 0.45	0.61 ± 0.36	1.18 ± 0.46
Ber (50 µmol/L)	1.47 ± 0.50	1.06 ± 0.24	0.85 ± 0.07
PDB + Ion	88.49 ± 1.91	89.30 ± 3.20	59.56 ± 5.69
PDB + Ion + Ber (100 µmol/L)	34.56 ± 5.21*	52.84 ± 3.93**	13.72 ± 1.13*
PDB + Ion + Ber (50 µmol/L)	63.43 ± 8.42*	79.03 ± 6.87	30.68 ± 4.01*
PDB + Ion + Ber (25 µmol/L)	72.14 ± 6.13	80.10 ± 10.44	40.35 ± 3.64*

Data are presented as mean $\pm$ SD (standard deviation) (n = 8). *$p < 0.01$ *vs* PDB + Ion; ** $p < 0.05$ *vs* PDB + Ion.

Table 2. The suppressive effect of berberine on the expression of CD69 or CD25 on T lymphocytes stimulated by PHA

Group	$CD3^+CD69^+/CD3^+$ (%)	$CD3^+CD25^+/CD3^+$ (%)
Control	0.61 ± 0.36	1.18 ± 0.46
Ber (50 µmol/L)	1.06 ± 0.24	0.85 ± 0.07
PHA (10 µg/ml)	39.44 ± 4.01	21.62 ± 2.27
PHA + Ber (100 µmol/L)	6.47 ± 0.62*	0.74 ± 0.29*
PHA + Ber (50 µmol/L)	20.94 ± 4.08*	5.32 ± 1.95*
PHA + Ber (25 µmol/L)	34.58 ± 3.39	12.37 ± 2.24*

Data are presented as mean $\pm$ SD (n = 8). *$p < 0.05$ *vs* PHA.

lymphocytes (11), was relatively low in the resting state of human peripheral T lymphocytes. In response to PDB plus ionomycin, CD69 expression was quickly up-regulated and the expression rate of $CD69^+$ T lymphocytes was around 85% of $CD3^+$ lymphocytes stimulated for 4 h (Figures 1A and 1C). In the presence of berberine, however, CD69 expression was significantly reduced and the inhibitory effect of berberine was dose dependent (Table 1 and Figure 1). Berberine alone showed no activation effect on T lymphocytes. As the incubation time was prolonged to 22 h, the inhibitory effect decreased (Table 1). It suggested that the effect of berberine on the activation of T lymphocytes in response to PDB plus ionomycin stimulation was partially reversible.

When the peripheral blood cells were stimulated by PHA for 22 h, the expression rate of CD69 was increased from baseline to 39%. Similarly, berberine significantly suppressed the expression of CD69 on T lymphocytes in a dose-dependent manner (Table 2).

Suppression of CD25 expression on T lymphocytes in the presence of berberine
CD25, the subunit of interleukin 2 receptor, is a middle-phase activation antigen of T lymphocytes. It was up-regulated in response to the stimulation of PDB plus ionomycin or PHA, while berberine alone did not have any effect on CD25 expression. Similar to the effect on CD69 expression, berberine could inhibit the CD25 up-regulation caused by PDB plus ionomycin or PHA alone (Tables 1 and 2).

To investigate whether berberine is cytotoxic to peripheral lymphocytes, the cells were stained with 7-AAD and cell death rates were analyzed with flow cytometry.
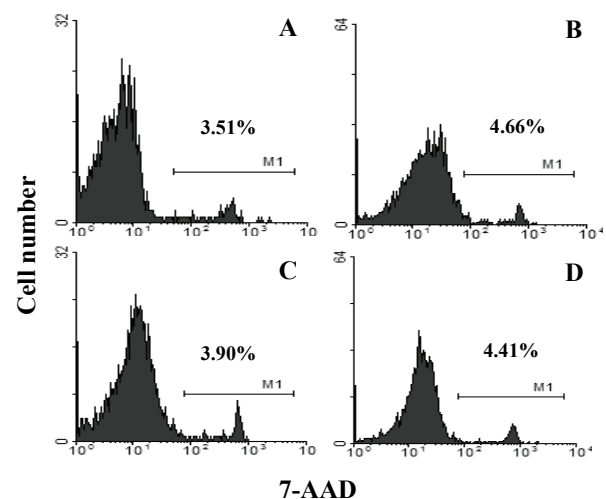

Figure 2. Effect of berberine (22 h incubation) on cell death rates of lymphocytes using 7-aminoactinomycin D staining. The values in each panel represent the rates of death cells (within M1 region). No significant difference was observed among these groups. Panel A, control; Panel B, Ber (50 µmol/L); Panel C, PDB + Ion; Panel D, PDB + Ion + Ber (50 µmol/L).
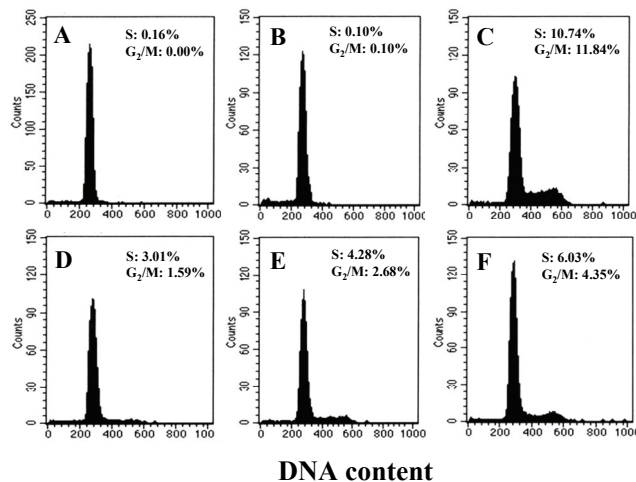
DNA content

Figure 3. Suppression of cell cycle progression of human peripheral lymphocytes by berberine. One representative experiment is showed and values in each panel are the percentage of lymphocytes in S phase and G_2/M phase respectively. Panel A, control; Panel B, Ber (50 μmol/L) alone; Panel C, PDB + Ion; Panel D, PDB + Ion + Ber (100 μmol/L); Panel E, PDB + Ion + Ber (50 μmol/L); Panel F, PDB + Ion + Ber (25 μmol/L).

Table 3). Furthermore, the apoptotic peaks (sub-G_0/G_1 peak) did not be affected by berberine (Table 3), suggesting that berberine arrested the lymphocytes at G_0/G_1 phase without inducing apoptosis of peripheral lymphocytes in the presence of PDB plus ionomycin. The results also showed that the berberine could prevent both the entrance of G_0/G_1 to S phase and G_2/M to S phase, indicating no phase specificity.

Discussion

The activation of T lymphocytes is accompanied by the up-regulation of a variety of surface antigens, such as CD69 and CD25, which are not expressed or only expressed at very low levels at resting state (11, 12). These newly expressed antigens are usually called activation antigens and now widely used as the activation markers for lymphocytes (13). CD69 is one of the earliest surface markers which are induced on T lymphocytes following their activation by mitogens, cytokines or contact with antigen-presenting cells (11, 13, 14). Although its ligand has not been identified, it plays an important part in signaling of T cell activation (13). The activation antigen CD25 is the subunit of interleukin 2 receptor and forms high affinity receptor together with β and γ subunits, thus it is critical for T lymphocyte proliferation (12). The present study showed that berberine could markedly suppress the expression of early activation antigen CD69 on T lymphocytes under stimulation of PHA alone or PDB plus ionomycin, and the inhibitory effect was dose-dependent and partially reversible. It could also inhibit the up-regulation of CD25, with no significant lymphotoxicity at the same concentration. Furthermore, berberine was able to substantially prevent cell cycle progression of lymphocytes in response to mitogens or polyclonal activators. Therefore, these results suggest that the alkaloid berberine suppresses the activation of T lymphocytes by inhibiting the expression of pivotal signaling molecules at the early phase of activation, which may subsequently interfere with other cell behaviors such as proliferation and differentiation. Since T lymphocytes play a central role in innate immunity and adaptive immunity, our results support such a concept that interference with early activation of T lymphocytes may be involved in the immunosuppressive effect of berberine.

7-AAD only stains dead cells which will be displayed as positive cells. The results showed that incubation of the cells with berberine (50 μmol/L) for 22 h failed to increase the death rates of lymphocytes (Figure 2), suggesting that the inhibitory effect on the activation of T lymphocytes was not due to its cytotoxic effect.

The effect of berberine on the cell cycle distribution of lymphocytes
Most of the unstimulated lymphocytes were at G_0/G_1 phase and only very small number of cells at S or G_2/M phases. Besides, there were about 1% cells distributed at sub-G_0/G_1 peak, the apoptotic peak. After stimulation with PDB plus ionomycin for 72 h, there were 10% and 8% of lymphocytes at S phase and G_2/M phase, respectively. Berberine inhibited the progression of cell cycles from G_0/G_1 phase to S and G_2/M phases in a dose-dependent manner (Figure 3 and

Table 3. The effect of berberine on cell cycle distribution of lymphocytes stimulated by PDB plus ionomycin for 72 h

Group	Cell cycle distribution (%)			
	sub-G_0/G_1	G_0/G_1	S	G_2/M
Control	1.1 ± 0.7	97.6 ± 2.6	0.18 ± 0.16	0.02 ± 0.03
PDB + Ion	1.1 ± 1.0	80.1 ± 4.9	10.9 ± 1.6	8.1 ± 3.2
PDB + Ion + Ber (100 μmol/L)	3.5 ± 1.5	91.3 ± 1.9	$3.6 \pm 0.37*$	$1.8 \pm 0.6**$
PDB + Ion + Ber (50 μmol/L)	1.6 ± 0.5	91.2 ± 1.7	$4.4 \pm 1.1*$	2.9 ± 1.3
PDB + Ion + Ber (25 μmol/L)	1.0 ± 0.3	89.8 ± 3.2	$5.1 \pm 1.8*$	4.2 ± 1.9

Data are presented as mean ± SD (n = 8). *$p < 0.01$ *vs* PDB + Ion; ** $p < 0.05$ *vs* PDB + Ion.

In this study the effect of berberine was investigated in whole blood system which mimics the *in vivo* physiological environment and has some advantages as compared with purified lymphocytes. However, it would be possible that other cells in blood might mediate the effects of berberine on lymphocytes. In the present study, we believe that this possibility could be ruled out due to the following reasons. First, both PBD and ionomycin are strong pharmaceutical reagents that quickly upregulated CD69 expression as early as 4 h after stimulation and their effects are seldom influenced by soluble cytokines (15). Second, berberine alone activated neither lymphocytes nor other leukocytes. Therefore, our results suggest that berberine exerts its effect directly on the lymphocytes.

Then how did berberine inhibit the activation and proliferation of the lymphocytes? Previously, it is established that berberine can induce apoptosis in 3T3 cell line (16) and HL-60 cells *in vitro* (17). However, berberine did not increase the apoptotic peak in peripheral lymphocytes in our study. Thus other mechanism may be involved in the inhibitory effect on lymphocyte activation. The present study showed that berberine not only inhibited the stimulation effect of PHA that acts on the surface receptor such as CD3/TCR complex, but also impeded the action of pharmacological agents phorbol ester and ionomycin, which directly activates the intracellular signal transduction components protein kinase C-θ (PKCθ) (15) and increases calcium ion concentration in the cytosol, respectively. The expression of CD69 is mainly owing to the activation of PKCθ by phorbol ester (15). It can thus be presumed that the target of berberine should be on PKCθ or its downstream signaling components. As there is no evidence that berberine had any direct effect on PKCθ and one study revealed that berberine did not interfere with PKC mediated contraction of artery ring (18), a more reasonable explanation seems to be that berberine may interfere with signaling pathway downstream of PKC.

As mentioned above, both *in vitro* and *in vivo* studies demonstrated that berberine had suppressive effect on the immune response (5-7, 19). Our results are consistent with previous studies and provide new evidence for the inhibitory effect of berberine on T lymphocytes. Recently, it had been reported (8) that berberine may suppress the interaction between lymphocytes and endothelial cells by decreasing the expression of adhesion molecules on lymphocytes, and the recirculation pattern of lymphocytes may thus be changed. Studies on experimental autoimmune tubulointerstitial nephritis mice demonstrated that berberine had immuno-suppressive effect and decreased the intensity of pathological injuries (7). Besides, these researchers also found that berberine could cause reduction in the number of lympho-cytes in circulation, but the reasons underlying are not clear (7). In another study, berberine was shown to depress the development of adjuvant-induced arthritis and to inhibit the delayed-type hypersensitivity (6). In addition to these *in vivo* studies, *in vitro* study showed that berberine can markedly inhibit the proliferation of lymphocytes activated with PHA or Con A only when added within the first 24 hour and the

effect is not due to lymphotoxcicity (19). This is consistent with our results that the effect of berberine on early signal transduction may be critical for its inhibition of T lymphocyte activation and proliferation. It was also found that berberine could suppress the production of cytokines such as IL-2 and IL-6 in animal model (7). Taken together, our results and other previous studies suggest that the immunosuppressive property of berberine is largely due to its inhibitory effect on the activation and proliferation of lymphocytes, especially T lymphocytes.

Moreover, studies on tumor cells revealed that the activator protein-1 (AP-1) transcriptional activity is potently inhibited by berberine in a concentration-dependent manner (20, 21), suggesting that suppression of the transcription of genes which have an AP-1 binding site in the promoter region may involve in anti-tumor activity of this alkaloid. As it is known that AP-1 plays an important role in the up-regulation of CD69 and it is activated by PKCθ (17), we postulate that AP-1 may be the potential target of berberine in lymphocytes and that depression of this transcriptional factor may contribute in part to the inhibitory effect of berberine on T lymphocytes activation. However, further work still need to be done to verify this hypothesis.

In summary, the present study indicates that berberine has inhibitory effect on the early activation of T lymphocytes and the potential target may be downstream of PKC. The suppression of the upregulation of important activation antigen such as CD69 hampers the subsequent signaling events, resulting in the hindrance of proliferation, which is likely one of the possible mechanisms of immunosuppressive effect of berberine. Further investigation remains to be done to clarify whether berberine exerts direct effect on AP-1 or other signaling components in lymphocytes.

Acknowledgements

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