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Inhibition of In-vitro Lymphocyte Transformation by the Isoquinoline Alkaloid Berberine

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Abstract

Berberine is an isoquinoline alkaloid with multiple pharmacological actions, including an anti-inflammatory activity. The effects of berberine on in-vitro cellular proliferation of human peripheral lymphocytes stimulated with phytohaemagglutinin, concanavalin A and pokeweed mitogen were studied. Mononuclear cells were cultured in flat-bottomed 96-well microplates at 37°C for 96–144 h in the presence of one mitogen at different concentrations and the alkaloid at doses of 2·5 to 20 $\mu g\,mL^{-1}$. The mitogen-induced response of lymphocytes was evaluated from the extent of the incorporation of [^{3}H]thymidine into cells in-vitro.

A consistent and progressive inhibitory influence of berberine with increasing concentrations in culture was identified with all mitogens and was more pronounced with pokeweed mitogen. The effect of berberine was observed in phytohaemagglutinin (PHA)- and concanavalin A-activated lymphocytes when the drug was added during the first 24 h of culture, whereas the same effect occurred throughout the incubation period in pokeweed mitogen-stimulated cells. The viability of lymphocytes following treatment with the drug, as assessed by the trypan blue exclusion test, revealed no change when compared with the same untreated lymphocytes, indicating no lymphocytotoxic activity.

We conclude that some effects of berberine, especially its anti-inflammatory action, may arise in part from the inhibition of DNA-synthesis in activated lymphocytes.

The isoquinoline alkaloid berberine is widely distributed in a variety of plant families such as Berberidaceae, Magnoliaceae, Menispermaceae, Ranunculaceae, Papaveraceae, Lauraceae, Annonaceae and Rutaceae (Lewis & Elvin-Lewis 1977), has multiple pharmacological actions (for review see Simeon et al 1989) while being relatively nontoxic to man (Rabbani et al 1987). It has been widely used as an antibacterial, antiparasitic, antifungal, hypotensive, antitumoral and as an anti-inflammatory drug (Kimura & Koizumi 1953; Fujimura et al 1960; Amin et al 1969; Nishino et al 1986). Some studies show the interaction of berberine with DNA (Creasey 1979; Molero & Stocker 1981), mainly between A-T base pairs (Sethi 1983), a possible property responsible for its antitumoral activity in leukaemias (Kondo 1976; Sethi 1979). Since, to our knowledge, no research has been conducted on the influence of berberine on the proliferative response of lymphocytes to allergens, antigens, mitogens or other biological stimulants, we investigated the effects of berberine on the cellular blastogenesis of human peripheral blood lymphocytes in response to phytohaemagglutinin, concanavalin A and pokeweed mitogen, a well known test of cell-mediated immunocompetence, in order to detect immunoregulatory properties of the drug.

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Materials and Methods

Lymphocytes

Samples of peripheral venous blood from healthy adult individuals were collected into heparinized tubes. The plasma was separated and the mononuclear enriched leucocyte population isolated by the Ficoll-Hypaque gradient method (Boyum 1968). The cells were removed from the interphase and, after washing three times with 5% foetal calf serum (FCS, virus and mycoplasma screened, Cultilab) in RPMI/1640 (with glutamine, Gibco), were centrifuged for 5 min at 400 *g* and finally suspended in RPMI/1640. The final concentration of the cell suspension was 2×10^6 cells mL^{-1} medium. Viability of cells was determined by the trypan blue exclusion test and was always higher than 95%.

Response to mitogens

The details of the procedure have been reported previously (Wajner et al 1986). Briefly, flat-bottomed 96-well microtitre plates (Falcon) were prepared by adding 50 μL cell suspension (1×10^5 cells), 60 μL medium containing phytohaemagglutinin (0·25 to 5%, v/v, Difco), concanavalin A (12·5 to 125 $\mu g\,mL^{-1}$, Pharmacia) or pokeweed mitogen (0·1 to 1·0%, v/v, Welcome), 40 μL autologous plasma and 50 μL medium containing 2·5–20 $\mu g\,mL^{-1}$ berberine (Merck). Berberine was dissolved in medium. The total culture volume (200 μL) contained in addition 100 int. units mL^{-1} penicillin and 100 $\mu g\,mL^{-1}$ streptomycin. Control cultures were prepared identically but without the drug. The plates were covered with plastic lids and

wrapped in adhesive plate film, to prevent dehydration. The cultures were incubated in a humidified atmosphere of 5% CO_2 in air at 37°C for 96 h (phytohaemagglutinin and concanavalin A) or 144 h (pokeweed mitogen). Twenty hours before harvest, 50 μL medium containing 0·5 μCi of 6[^{3}H]-thymidine (TRA 306, Amersham, spec. act. 2 Ci mmol^{-1}) was added to each well. Cultures were harvested into fibre paper filters with a semi-automatic multiple harvester (Cambridge Technology Inc.). Filters were dried at 60°C for 60 min or overnight at 37°C. The area of the filter sheets which contained cells (discs) was pushed into plastic vials, and 5 mL scintillation fluid was added to each vial. Cell-bound [^{3}H]thymidine was determined by counting in a LKB liquid scintillation counter. Identical triplicate cultures were always performed and the median of each triplicate was used in the calculations. Comparison between means of lymphocyte responsiveness was calculated by analysis of variance followed by the Duncan's multiple range test or by the Student's t-test. Results were expressed as counts min^{-1} or percentage of control cultures.

Results

The responsiveness of lymphocytes under various experimental conditions is displayed in Table 1. Inhibitory activities on the incorporation of [^{3}H]thymidine were observed in cultures containing berberine, achieving 85% inhibition with 20 μg mL^{-1} for phytohaemagglutinin and concanavalin A-stimulated lymphocytes and with 10 μg mL^{-1} for pokeweed mitogen-treated cells. A gradual decrease in mean lymphocyte response with higher concentrations of berberine in the medium can also be observed.

Next, we added 10 μg mL^{-1} berberine at different times during incubation and compared the lymphocyte responses to the mitogens. In cultures supplemented with phytohaemagglutinin and concanavalin A, the inhibitory action of berberine occurred only when the drug was added during the first 24 h, whereas in cultures containing pokeweed mitogen the inhibition persisted even after 96 h from the start of culture (Table 2).

Cell viability in controls and in cultures containing 20 μg mL^{-1} berberine was practically the same (70–85%) at the end of cultures stimulated with the various mitogens (results not shown).

Table 1. Effect of the isoquinoline alkaloid berberine on the in-vitro proliferative response of human peripheral lymphocytes stimulated with phytohaemagglutinin, concanavalin A and pokeweed mitogen. The final concentrations of berberine and mitogen in the culture fluid are given in the Table. Results are means and standard error of the mean of four experiments (lymphocyte donors) performed in triplicate, expressed in counts per minute of [^{3}H]thymidine incorporation. Differences between means for the various groups were calculated by analysis of variance and by the Duncan multiple range test.

	Lymphocyte proliferation (cpm/10^3)		
Berberine (μg mL^{-1})	Phytohaemagglutinin (%) 0·25	1·0	5
Control	15·5 ± 5·2	31·0 ± 13	19·5 ± 8·6
5	5·4 ± 3·2**	18·2 ± 6·8**	14·9 ± 8·5**
10	4·9 ± 2·2**	8·5 ± 3·3**	6·0 ± 3·0**
20	2·7 ± 0·4**	3·5 ± 0·8**	2·8 ± 0·6**
	Concanavalin A (μg mL^{-1}) 12·5	50·0	125
Control	16·9 ± 3·0	18·9 ± 2·6	25·1 ± 11·6
5	10·3 ± 3·6**	12·7 ± 3·3**	17·4 ± 6·3**
10	5·1 ± 2·7**	7·1 ± 2·0**	7·9 ± 0·5**
20	3·3 ± 0·1**	3·5 ± 0·1**	3·7 ± 1·0**
	Pokeweed mitogen (%) 1·0	0·25	1·0
Control	15·5 ± 1·1	13·8 ± 3·4	8·4 ± 1·5
2·5	10·4 ± 4·0**	11·0 ± 2·5	6·2 ± 3·4
5	5·8 ± 2·7**	6·8 ± 2·5**	2·7 ± 1·7**
10	2·7 ± 0·9**	2·0 ± 0·1**	1·0 ± 0·6**

*P < 0·05, **P < 0·01 compared with the control group.

Discussion

Evidence has been presented in this study that there is a progressive and consistent inhibition of DNA synthesis in mitogen-activated lymphocytes, which is proportional to the concentration of berberine, in the incubation medium. This suppression is not fortuitous, since it occurs under various in-vitro culture conditions, using different mitogens which stimulate large groups of T cells (phytohaemagglutinin and concanavalin A) or predominantly B cells (pokeweed mitogen). The inhibition of lymphocyte blastogenesis was more pronounced in cultures supplemented with pokeweed mitogen, indicating that the response of B lymphocytes was more susceptible to the action of the alkaloid. On the other hand, we observed that increasing mitogen concentration did not give rise to an increase in the proliferation of lymphocytes immersed in medium containing berberine, indicating that a

Table 2. Inhibitory effects of berberine (10 μg mL^{-1}) added at different times during incubation on mitogen-induced lymphocyte proliferation. The values are expressed as percentage of lymphocyte responsiveness (mean ± s.d., n = 4 lymphocyte donors) of control cultures (100% growth) where no alkaloid was added. Differences between the means of controls and the other groups were calculated by the Student's t-test for paired samples.

	Lymphocyte proliferation (% control)		
Time (h)	Phytohaemagglutinin (1%)	Concanavalin A (50 μg mL^{-1})	Pokeweed mitogen (0·25%)
0	34 ± 6·7**	34 ± 4·0**	16 ± 8·6**
24	59 ± 17·7	77 ± 19·4*	24 ± 9·7**
48	100 ± 16·7	122 ± 9·0	24 ± 10·7**
72	135 ± 28·0	120 ± 10·7	49 ± 9·4**
96	—	—	70 ± 17·9**

*P < 0.05, **P < 0.01.

competition between berberine and the mitogens for a possible single receptor on the lymphocyte surface is unlikely.

We also verified that the action of berberine on phytohaemagglutinin-and concanavalin A-stimulated lymphocytes only occurred when the drug was present at the beginning of the incubation time, suggesting that its effect takes place early during the cell cycle. In contrast, in pokeweed mitogen-induced lymphocytes, the effect was evident even 96 h after the beginning of cultures, indicating early and late influence on the cell cycle.

Furthermore, the viability of lymphocytes treated with the highest concentration of berberine utilized in the cultures ($20\,\mu g\,mL^{-1}$) and induced to proliferate with all mitogens was similar to that of untreated lymphocytes. These results indicate that under the conditions of our assays, the alkaloid is not lymphocytotoxic but rather inhibitory towards DNA synthesis, reinforcing our findings of a consistent in-vitro immunosuppressive activity of berberine.

The mechanism by which berberine inhibits in-vitro lymphocyte growth is unknown. It may be due to berberine interaction with nucleic acids, mainly with polyadenylic and polyguanylic acids (Creasey 1979). In this context, we recently identified in our laboratory cytostatic effects caused by berberine in dividing haploid and diploid *Saccharomyces cerevisiae* cells (Pasqual et al 1993). However, these effects were mild at $25\,\mu g\ mL^{-1}$ berberine, only becoming significant at concentrations of $50\,\mu g\,mL^{-1}$ or higher. In our experiments $10\,\mu g\,mL^{-1}$ already caused an average cell growth suppression of 70 to 90%.

An alternative explanation for the inhibitory action of berberine on in-vitro lymphocyte proliferation might be the competition between the drug and glucose uptake, described in sarcoma 180 tumour cells (Creasey 1979; Nishino et al 1986), possibly leading to a secondary energy lymphocyte deprivation. This is however unlikely, since competition between the drug and glucose for membrane transport would not explain the strong activating property of berberine on peritoneal macrophages. Macrophages become cytotoxic to EL4 tumour cells in-vitro in the presence of the alkaloid (Kumazawa et al 1984), and are thought to explain the antimicrobial and antitumoral action of berberine-type alkaloids (Amin et al 1969; Nishino et al 1986; Rabbani et al 1987).

In conclusion, we found that berberine inhibits DNA synthesis of mitogen-activated human peripheral lymphocytes. Although our results should be interpreted cautiously before further evidence is obtained using other in-vitro and in-vivo systems, our findings support some properties attributed to berberine and more specifically its anti-inflammatory action.

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References

Amin, A. H., Subbaiah, T. V., Abbasi, K. M. (1969) Berberine sulfate: antimicrobial activity, bioassay and mode of action. Can. J. Microbiol. 15: 1067–1076

Boyum, A. (1968) Isolation of leukocytes from human blood: further observation. Scand. J. Lab. Invest. 21: 31–50

Creasey, W. A. (1979) Biochemical effects of berberine. Biochem. Pharmacol. 28: 1081–1084

Fujimura, H., Sawada, T., Goto, N. (1960) Screening test of antiinflammatory action of the constituents of crude drugs and plants. J. Pharmacol. Soc. Japan 90: 782–783

Kimura, Y., Koizumi, S. (1953) Studies on the standardization of crude drugs II. On the production conditions and component of crude drugs (I), on the production conditions and berberine content of Phellodendron. J. Pharmacol. Soc. Japan 73: 194–195

Kondo, Y. (1976) Organic and biological aspects of berberine alkaloids. Heterocycles 4: 197–219

Kumazawa, Y., Itagaki, A., Fukumoto, M., Fujisawa, H., Nishimura, C., Nomoto, K. (1984) Activation of peritoneal macrophages by berberine-type alkaloids in terms of induction of cytostatic activity. Int. J. Immunopharmacol. 6: 587–592

Lewis, W. H., Elvin-Lewis, M. P. F. (1977) Internal poisons. In: Manske, R. H. F., Holmes, H. L. (eds) Alkaloids: Chemistry and Physiology. Academic Press, New York, vol 4, pp 77–118

Molero, M. L., Stocker, J. C. (1981) Fluorescence reaction of chromatin and basophilic cytoplasm by berberine sulfate. Cell Mol. Biol. 27: 523–525

Nishino, H., Kitagawa, K., Fujiki, H., Iwashima, A. (1986) Berberine sulfate inhibits tumor-promoting activity of teleocidin in two-stage carcinogenesis on mouse skin. Oncology 43: 131–134

Pasqual, M. S., Lauer, C. P., Moyna, P., Henriques, J. A. P. (1993) Genotoxicity of isoquinoline alkaloid berberine in prokaryotic and eukaryotic organisms. Mutat. Res. 286: 243–252

Rabbani, G. H., Butler, J., Knight, J., Sanyal, S. C., Alam, K. (1987) Randomized controlled trial of berberine sulfate therapy for diarrhea due to enterotoxigenic *Escherichia coli* and *Vibrio cholerae*. J. Infect. Dis. 155: 979–984

Sethi, M. L. (1979) Inhibition of reverse transcriptase activity by benzophenanthridine alkaloids. J. Nat. Prod. 42: 187–196

Sethi, M. L. (1983) Enzyme inhibition VI: inhibition of reverse transcriptase activity by protoberberine alkaloids and structure-activity relationship. J. Pharm. Sci. 72: 538–541

Simeon, S., Rios, J. L., Villar, A. (1989) Pharmacological activities of protoberberine alkaloids. Plant Med. Phytother. 23: 202–250

Wajner, M., Papiha, S. S., Wagstaff, T. I. (1986) Inhibition of mitogen and allogeneic stimulated lymphocyte growth by human amniotic fluid: lack of correlation between α-fetoprotein and in vitro immunosuppression. Eur. J. Obstet. Gynecol. Reprod. Biol. 21: 225–232