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BIOCHEMISTRY OF THE EFFECTS OF ASIATICOSIDE ON HUMAN POLYMORPHONUCLEAR LEUKOCYTES

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Abstract—Asiaticoside is found to increase the $^{14}CO_2$ production from glucose-U-^{14}C both in resting and phagocytizing human polymorphonuclear leukocytes; but does not seem to affect the methylene blue or cyanide stimulated $^{14}CO_2$ production at one or other of these physiologic states. Inhibition of $^{14}CO_2$ production by hydrocortisone is increased through asiaticoside, however, especially when leukocyte H_2O_2 production is at its maximum. Asiaticoside also relieved the 2-deoxyglucose inhibition partially, both at rest and during phagocytosis. These findings were interpreted as indications of the possible action of asiaticoside on leukocyte membrane. When compared with the action mechanisms of other membrane-affecting surface active agents, the effects summarized above, could be explained by the saponin-like structure of asiaticoside.

ASIATICOSIDE (O-α-L-rhamnopyranosyl-(1 → 4)-O-β-D-glucopyranosyl-(1 → 6)-O-β-D-glucopyranose-1-asiaticat) preparations have been used as bactericidal agents and to promote wound healing in the past.[1] Although the pharmacological action of asiaticoside has been documented, no detailed biochemical investigation on its action mechanism has yet been carried out,[2] and its cicatrization increasing effect has been proposed to depend upon the action of this compound upon the reticuloendothelial system.[1] The interesting pentacyclic triterpene structure, somewhat resembling other saponins and steroids, has raised the question of whether the action of asiaticoside is similar to other saponins and to other steroid hormones or not.[3]

Since the effects of saponins, steroid hormones, phagocytosis, surface active agents and various other effectors upon leukocyte carbohydrate metabolism are well documented,[4–7] the leukocyte was selected for the study of the effects of asiaticoside. Our experiment showed that asiaticoside stimulated $^{14}CO_2$ production from glucose-U-^{14}C, both in resting and phagocytizing leukocytes which indicates the stimulation of hexosemonophosphate (HMP) shunt, as the origin of $^{14}CO_2$ production from glucose-U-^{14}C has been definitely linked to this metabolic pathway in leukocytes.[4] This HMP shunt stimulating effect, has been linked to its saponin-like structure. Saponins and other surface active agents have also been shown to stimulate the leukocyte $^{14}CO_2$ production.[6] The postulated action mechanism is supported further by asiaticoside's potentiating the hydrocortisone (HC) inhibitory effect, and by its relieving action on the 2-deoxyglucose (2DG) inhibition; asiaticoside on the other hand has no effect upon methylene blue (MB) or cyanide (CN) stimulation of the leukocyte HMP shunt.

MATERIALS AND METHODS

Preparation of leukocytes. Blood was obtained from normal, healthy, Blood bank donors; leukocytes were separated by using Dextran and EDTA.[8] In the final preparation, the erythrocyte contamination was reduced to 2–4 per cent by 20 sec osmotic shock.[9] Modified Hank's balanced salt solution (HBSS)[8] was used to suspend the leukocytes. The method employed yielded a nearly pure preparation of polymorphonuclear (PMN) leuckocytes (87 ± 11 per cent). Plastic or siliconized glassware was used for leukocyte preparation. The assays were carried out in 25 ml siliconized Warburg flasks. The center well contained 0·45 ml of 20 per cent NaOH; gas phase was room air. The 2 ml incubation medium consisted of HBSS containing 0·5–1 $\times$ 10^7 leukocytes/millilitre, 5 $\times$ 10^{-3} M glucose, 2 μc of G-U-^{14}C (specific activity 0·2 mc/mmole), 10 per cent (v/v) donor's serum, 0·15 per cent (v/v) Dimethylformamide (DMFA)* at pH 7·7 and also the compound under study. Following the addition of MB, CN, asiaticoside etc., a preliminary incubation in the absence of glucose-U-^{14}C was carried out for 30 min at 37° in a Dubnoff metabolic shaker. When leukocytes were stimulated for phagocytosis, polystyrene latex particles, 0·714 μ diameter, were added to obtain a final concentration of 1·25 $\times$ 10^9 particle per ml.

The incubation was terminated with the addition of 1 ml of 0·5 N H_2SO_4 and the liberated $^{14}CO_2$, trapped by NaOH was counted using 10 ml of counting solution (Naphthalene 80 g, 2,5-diphenyloxazole (PPO) 4 g, 1,4-bis [2(4-methyl-5-phenyloxazolyl)] benzene (dimethyl POPOP) 0·2 g, per l. of toluene and absolute ethanol 60/40 (v/v) for 0·15 ml NaOH). A Packard liquid scintillation counter, Model 3380 (with AAA 540), was employed to obtain the absolute activities. Since asiaticoside is insoluble in this incubation medium, a carrier solvent has to be used, and DMFA proved suitable for this purpose. Concentrations of DMFA higher than 0·3 per cent however, inhibited leukocyte $^{14}CO_2$ production. Therefore an attempt was made not to exceed a DMFA concentration over 0·15 per cent, making the testing of the asiaticoside effect impossible at higher concentrations. In order to minimize the effect of biological dispersion and to obtain valid statistical evaluation, the experiments were designed so that the controls could be run simultaneously with the compounds tested, viz. using the same donor's leukocytes. Standard biostatistical methods were used to calculate the results.[10]

Asiaticoside was a gift of Laboratories La Roche, Levallois, France and hydrocortisone succinate (Solu-cortef) was obtained from Upjohn International Inc., Mich., U.S.A.

RESULTS

The effect of asiaticoside. Asiaticoside stimulated $^{14}CO_2$ production from G-U-^{14}C, in resting and phagocytizing leukocytes (Fig. 1). The degree of this stimulation was about the same in both groups, and could be observed at relatively lower concentrations of the drug.

The effect of asiaticoside upon the action of other compounds. Leukocyte $^{14}CO_2$ production from G-U-^{14}C, could be stimulated by the addition of MB and CN, or it could be inhibited by the presence of HC and 2DG. The effects of the latter compounds at their optimum concentration together with the effect of asiaticoside are shown in

* See below the reason of choice for DMFA.

resting (Fig. 2a) and phagocytizing (Fig. 2b) leukocytes. The MB, $^{14}CO_2$ production stimulation was a nonspecific process, evidenced by its being of about the same in magnitude regardless of the stimulation of the leukocyte by the latex particles or by the presence of CN; the MB stimulation could be inhibited by HC however. Asiaticoside had no effect upon the MB stimulation with or without other compounds mentioned above. The addition of CN also led to an increase in $^{14}CO_2$ production and

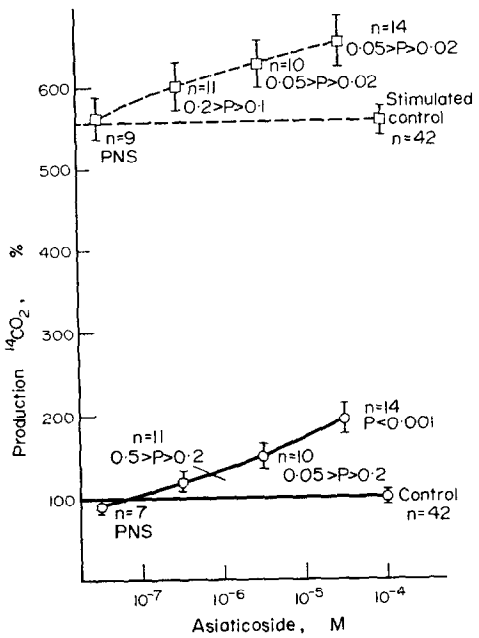

FIG. 1. The effect of asiaticoside on leukocyte $^{14}CO_2$ production from G-U-^{14}C. The asiaticoside concentration is shown on the abscissa. The rates are expressed on the ordinate as per cent $^{14}CO_2$ production change observed as compared to the unstimulated control leukocytes: Control leukocytes uptake = 7·11 ± 0·71 μM glucose/10^{10} leukocytes, per hr. Unstimulated leukocytes, ○——○; latex particle stimulated leukocytes, □——□. All experiments are reported as mean ±S.E.M. and (n) denotes the number of experiments, P values, as indicated.

this effect was observed both in resting and stimulated leukocytes. The presence of asiaticoside did not affect the stimulation caused by CN either. Addition of HC inhibited $^{14}CO_2$ production, and the presence of asiaticoside now potentiated the effect of HC; this was best manifested when latex-stimulated leukocytes were used ($0{\cdot}01 < P < 0{\cdot}02$). When 2DG was added, $^{14}CO_2$ production was inhibited both in resting and stimulated leukocytes; the addition of asiaticoside relieved this inhibition in both physiologic states ($0{\cdot}02 < P < 0{\cdot}05$).

DISCUSSION

The anaerobic glycolytic pattern of the leukocyte has been found to be stimulated by phagocytosis, evidenced by increased lactate production and stimulated HMP shunt, i.e. increased $^{14}CO_2$ production from glucose-U-^{14}C.[4,11] As this stimulation

was due to a multi-step metabolic process, varied compounds were found to be affecting it; their action proved to be at more than one site of this complex triggering mechanism, starting with the stimulation of leukocyte membrane (surface active agents),[6,12] and continuing with the adhesion of the leukocyte membrane and particles (2DG, EDTA),[13] increased H_2O_2 production, with the regulation of reduced nucleotide oxidation (MB and CN)[7,11,14] and oxidases[15] (HC). The stimulation of leukocyte $^{14}CO_2$ production from glucose-U-^{14}C by asiaticoside, may be due to any one of the above factors.

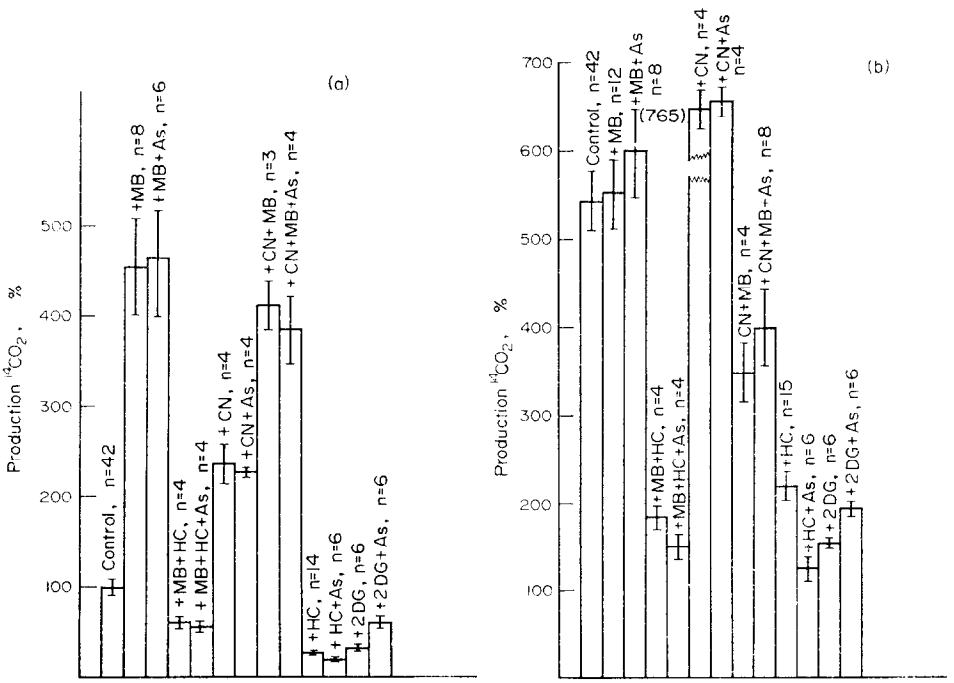

FIG. 2. The effect of asiaticoside and various compounds upon $^{14}CO_2$ production. (a) resting leukocytes, (b) leukocytes were stimulated by latex particles. Rates are shown on the ordinate as per cent $^{14}CO_2$ production compared to the unstimulated control leukocytes which value is reported in the experiments given in Fig. 1. Experimental conditions are as reported. 0·15% DMFA was present in all experiments. Control (C); 5×10^{-5} M methylene blue present (MB); 3×10^{-5} M asiaticoside added (As); $2{\cdot}1 \times 10^{-3}$ M hydrocortisone succinate present (HC); 10^{-3} M NaCN present (CN); 10^{-3} M 2-deoxyglucose present (2DG). All experiments are reported as mean ±S.E.M. and (n) denotes the number of experiments.

The effect of MB is thought to occur through increased nonspecific oxidation of NADPH, and to reflect the total HMP shunt capacity of the cell.[4,16] As asiaticoside failed to change the effect of MB in the presence or absence of other compounds, the insensitivity of HMP shunt enzymes to asiaticoside should be presumed. The addition of CN which inhibits the usual oxidative breakdown of glucose has been shown to stimulate the $^{14}CO_2$ production in leukocytes.[4] The stimulatory effect is thought to occur through increased NADPH availability to a CN insensitive CO_2 producing system, namely, glutathione peroxidase and glutathione reductase.[14] Asiaticoside

failed to affect the CN stimulation, possibly indicating a lack of its direct interaction with glutathione peroxidase or glutathione reductase systems.

The inhibitory effect of 2DG upon the ingestion of polystrene particles is explained by an effect of this compound upon leukocyte adhesion to particles;[13] 2DG has also been shown to inhibit the transport and utilization of glucose in various organs.[18] Hence the Glucose-U-$^{14}C \rightarrow {}^{14}CO_2$ production inhibitory effect of 2DG, in either resting or phagocytizing leukocytes is to be anticipated. Asiaticoside presence was found to relieve this inhibition partially, both in resting and stimulated leukocytes, hence this effect might be due to an activating effect of this compound on leukocyte membrane, leading to an increased glucose uptake. When HC was present, the $^{14}CO_2$ production was strongly inhibited. This effect of HC has been related to its being a general oxidase inhibitor.[15] The inhibitory action of HC on MB stimulation could be explained by the sensitivity of NADPH-MB electron transport system or of HMP shunt[15,17] to HC. When asiaticoside was present, it potentiated the effect of HC, especially when leukocytes were stimulated; hence H_2O_2 production was at its maximum level.[4] If an activating effect of asiaticoside on membrane is postulated, increased HC penetration and potentiation of HC effect should be expected.

Surface active agents as saponins, digitonin and deoxycholate are also shown to affect the leukocyte glycolytic mechanism; the effective concentration of asiaticoside is within the range described for the above-mentioned compounds.[6,12] The action of these latter compounds upon glycolysis is attributed to their surface active properties, and is considered to occur primarily through an excitation of cell membrane.[12] Although saponin is found to stimulate the NADPH oxidase, the H_2O_2 producing key enzyme, the intracellular effect of this surface active agent is thought to occur secondarily to its primary membrane action; e.g. the saponin effect upon NADPH oxidase is observed only in intact cells; upon disruption, this effect disappears.[6,7] These compounds are thought to mimic the phagocytic stimulus;[7] and as the action of endotoxins of lipopolysaccharide structure is similar. This effect is considered as an indication of the basis of the initiating step in phagocytosis. However, not all membrane-activating compounds have similar mechanisms of action; for instance, endotoxin stimulates glycolysis, but has no effect upon G-1-$^{14}C \rightarrow {}^{14}CO_2$ production; whereas digitonin, deoxycholate and saponin inhibit glycolysis and also stimulate the $^{14}CO_2$ production, which is a process insensitive to CN, rotenone and antimycin A.[6] The recent work of Woodin[19] has indicated the complex ionic movement, the release of granular components and the effect of Ca^{2+} as related to the action of leucocidin on polymorphonuclear leukocytes proceeding through an interaction of this toxic protein with the PMN leukocyte membrane possibly its modification of the triphosphoinositide.[20]

The results stated in this study, support and extend the findings reported for surface active agents, and suggest a mode of action for asiaticoside similar to that of other saponins. The HMP shunt stimulating action, the potentiation of HC effect under physiological conditions, and the partial relief of 2DG inhibition, all produced by asiaticoside, might also be due to the primary action of asiaticoside on the leukocyte membrane.

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