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Biphasic and Differential Effects of the Cytostatic Agents Avarone and Avarol on DNA Metabolism of Human and Murine T and B Lymphocytes*

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Abstract—*The two novel antimitotic and potent antileukemic agents avarone and avarol were determined to inhibit the [^{3}H]-dThd incorporation rates of both murine spleen and human peripheral blood lymphocytes within the concentration range of* 2–6 μM. *The mitogens concanavalin A (ConA; for T lymphocytes), lipopolysaccharide (LPS; for murine B lymphocytes) and pokeweed mitogen (PWM; for human T and B lymphocytes) were used to stimulate DNA synthesis in the lymphocyte fractions. The* ED_{50} *concentrations, causing a* 50% *reduction of [^{3}H]-dThd incorporation, were significantly lower in the experiments with avarone than in those with avarol. Moreover it was established that the DNA synthesis of ConA-activated lymphocytes was more sensitively inhibited by the compounds than that of LPS or PWM-activated cells, or non-activated cells. In addition it was elucidated that at low concentrations* (1–2 μM) *avarone and avarol caused a stimulation of dThd incorporation only in LPS or PWM-activated lymphocytes. Based on these results it is assumed that both antileukemic agents also affect differentially the different hematologic neoplasms.*

INTRODUCTION

BASIC STUDIES to elucidate the symbiotic relationship between sponges and their species-specific bacterial flora led to the discovery of the sesquiterpenoid quinone avarone and its hydroquinone derivative avarol [1, 2]. These two secondary metabolites from the marine sponge *Dysidea avara* were determined to be inhibitors of mitosis of eukaryotic cells [2] possibly due to an interference of these compounds with the process of tubulin polymerization [3]. Recently we noticed that both avarone and avarol exhibit potent antileukemic activity not only *in vitro* [2] but also *in vivo* [4]. An interesting feature of these cytostatic agents is their highly selective inhibitory activity on lymphocytes *in vitro* [4]. Dose–response experiments with the T cell lymphoma cell line L5178y [5] revealed that avarone inhibits cell growth at 0.62 μM and avarol causes the same effect at 0.93 μM [4]. The corresponding ED_{50} concentrations in *in vitro* experiments with non-lymphoid cells (e.g., melanoma cells, HeLa cells, human fibroblasts and human gingival cells) were determined to be 15–120-fold higher.

In the present study we determined the influence of avarone and avarol on normal lymphocytes *in vitro*. In the first section of this paper, the influence on the mitotic index was studied. In the second part, the effect on DNA synthesis in T and B lymphocytes was investigated. As mitotic inductors we used concanavalin A [ConA] for T lymphocytes from both mouse spleen and human peripheral blood [6], lipopolysaccharide [LPS] for murine B lymphocytes [7] and pokeweed mitogen [PWM] for human T and B lymphocytes [6]. The results revealed that avarone and avarol preferentially inhibit DNA synthesis in T cells. Moreover it was found that at non-cytostatic concentrations the two compounds enhance DNA synthesis in B lymphocytes.

MATERIALS AND METHODS

Materials

Concanavalin A [ConA] (no. C 5275), lipopolysaccharide [LPS] (no. L 4130) and pokeweed

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mitogen (no. L 9379) were obtained from Sigma, St. Louis, MO (USA); [methyl-^{3}H]thymidine (spec. act. 87 Ci/mmol) from the Radiochemical Centre, Amersham, U.K.; colcimide from Ciba–Geigy, Wehr, W. Germany and phytohemagglutinin (PHA 15) from Deutsche Wellcome, Burgwedel, W. Germany.

Avarol was isolated from *Dysidea avara* (2), which was collected in the Bay of Kotor (Yugoslavia). Avarone was obtained from its corresponding hydroquinone avarol by Ag_2O oxidation (2).

Cultivation of lymphocytes from mouse spleen

Spleen lymphocytes were prepared from 5–6-week-old male outbred NMRI mice as described [8]. The erythrocyte-free and macrophage-containing lymphocytes (2.5×10^6 cells) were placed in a final volume of 200 µl on microtiter plates and incubated for 72 hr in Dulbeccos minimum essential medium (DMEM), supplemented with 2 mM glutamine and 10% fetal calf serum. Eighteen hours prior to the end of the incubation 0.1 µCi of [^{3}H]-dThd was added to each cup. Where indicated 2 µg/ml of ConA or 20 µg/ml of LPS were added to the cultures. The cytostatic agents avarone and avarol were dissolved in dimethyl sulfoxide and added at time zero to the cultures. The final concentration of dimethyl sulfoxide was 0.1%; at this concentration dimethyl sulfoxide was found not to influence the [^{3}H]-dThd incorporation rate. Incorporation of [^{3}H]-dThd was determined as described [9].

Each value came from six parallel experiments. The ED_{50} concentrations causing a 50% reduction of [^{3}H]-dThd incorporation were estimated by logit regression [10].

Preparation of human lymphocytes from circulating blood

From freshly drawn heparinized blood (10 IU of heparin sodium salt/ml blood) the lymphocytes were isolated according to the described procedure by Ficoll-Ronpacon [11]. The resulting lymphocytes were suspended at a density of 4×10^6 cells/ml DMEM, supplemented with 10% fetal calf serum. 1×10^6 cells in 200 µl were placed on microtiter plates and processed as described above. The cultures were incubated either in the absence or presence of mitogen (2 µg/ml of ConA or 3 µg/ml of PWM).

Determination of mitotic index

Purified human peripheral blood lymphocytes were incubated for 70 hr in DMEM supplemented with 10% fetal calf serum at a density of 1×10^6 cells/ml in the presence of colcimide (0.1 µg/ml) and phytohemagglutinin (2 µg/ml). During the last 3 hr of incubation, different concentrations of avarol were added to the 5-ml assays. The cells were then processed as follows: 20 min exposure to hypotonicity achieved by adding 3 vol. of distilled water to the cultures; fixation was in 60% acetic acid-0.1 *N* hydrochloric acid for 15 min; staining with 2% acetic orcein. Squash preparations were made on siliconized slides.

For the determination of the mitotic index, 1000 to 1500 cells were analyzed per assay.

Statistical evaluation

t-tests to determine the significance of the growth inhibition effects in the presence or absence of mitogens were performed according to Student [10].

RESULTS

Increase of mitotic index by avarol treatment

After a 3-hr incubation of peripheral blood lymphocytes in the presence of avarol, the mitotic index substantially increased. At drug concentrations of 1.5, 3.0 or 6.0 µM the mitotic index increased to 6.27, 8.91 or 9.45. The mitotic index of the controls (absence of avarol) was 3.51.

Effect of avarone and avarol on mouse spleen lymphocytes

In the absence of mitogen the [^{3}H]-dThd incorporation rate in the lymphocyte cultures was determined to be $1.3 \pm 0.1 \times 10^3$ dpm/2.5×10^6 cells per 18 hr. Addition of 2 µg ConA/ml or 20 µg LPS/ml to the cultures augmented the incorporation rate to $21.7 \pm 1.9 \times 10^3$ dpm or $45.8 \pm 3.6 \times 10^3$ dpm/2.5×10^6 cells per 18 hr respectively.

As summarized in Fig. 1, avarone was the more potent inhibitor of DNA synthesis (measured by the [^{3}H]-dThd incorporation rate) than avarol irrespectively of the activation state of the lymphocytes. The following ED_{50} concentrations were estimated from the dose–response experiments; for avarone: non-activated cultures, 2.9 ± 0.2 µM; ConA-stimulated lymphocytes, 1.9 ± 0.2 µM; and LPS-stimulated cells, 4.3 ± 0.3 µM; and for avarol: non-activated cultures; 3.8 ± 0.3 µM; and ConA- or LPS-stimulated lymphocytes, 2.4 ± 0.2 µM and 5.9 ± 0.4 µM, respectively. In all three cases the differences in the inhibitory potencies between avarone and avarol were statistically significant (*P*-value: ≤ 0.001).

Interesting was the finding (Fig. 1) that both avarone and avarol at low concentrations enhanced DNA synthesis in non-activated lymphocytes and especially in LPS-stimulated lymphocytes. The highest stimulatory effect was measured in the drug ranges 1–2 µM; maximal stimulation for avarone was determined to be 203% (control: 100%; *P* value vs. control ≤ 0.001) at a concentration of 1.8 µM, and for avarol, 168% (*P*: ≤ 0.001) at the same concentration.

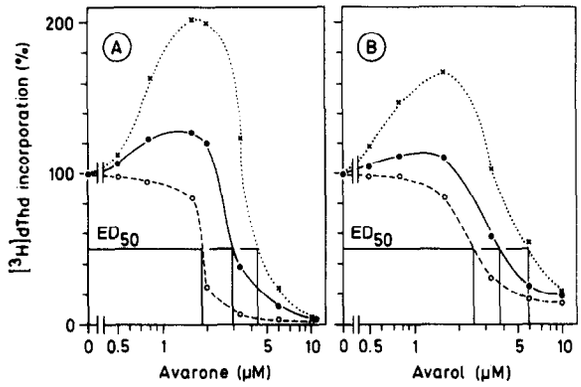

Fig. 1. Effect of avarone (A) *and avarol* (B) *on* [^{3}H]*-dThd incorporation into murine spleen lymphocytes in the absence* (●—●), *or the presence of* 2 μg *concanavalin* A/ml (o--o) *or* 20 μg *lipopolysaccharide/*ml (×····×). *Addition of* [^{3}H]*-dThd*: 18 hr *prior to the end of the incubation. Avarol or avarone was added at time zero. Means of quadriplicate experiments are presented; the S.D. varied between* 6 *and* 8%. *The values for a* 50% *inhibition of* [^{3}H]*-dThd incorporation of the corresponding dose–response experiments are given as vertical lines.*

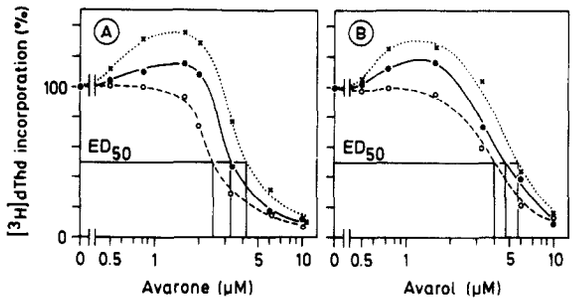

Fig. 2. Effect of avarone (A) *and avarol* (B) *on* [^{3}H]*-dThd incorporation into human peripheral blood lymphocytes in the absence* (●—●), *or the presence of* 2 μg *concentration* A/ml (o----o) *or* 3 μg *pokeweed mitogen/*ml(×····×). *Further details are given in the legend to Fig.* 1.

Effect on human peripheral blood lymphocytes

The basis incorporation rates in the human lymphocyte cultures were as follows; without mitogen, 1.4 ± 0.2 × 10^3 dpm/l × 10^6 cells per 18 hr and in the presence of 2 μg ConA/ml or 3 μg PWM/ml, 17.4 ± 1.4 × 10^3 dpm or 35.9 ± 2.6 × 10^3 dpm/l × 10^6 cells per 18 hr, resp.

As already determined in the experiments with mouse spleen cells, avarone exhibited a significantly higher inhibitory potency (P: ≤ 0.001) also on human lymphocytes than avarol. The following ED_{50} values were determined for avarone (avarol): non-activated lymphocytes, 3.2 ± 0.3 (4.9 ± 0.4) μM; ConA-stimulated cells, 2.3 ± 0.2 (4.0 ± 0.3) μM and PWM-stimulated cells, 4.2 ± 0.3 (5.8 ± 0.4) μM (Fig. 2).

As in the experiments with murine lymphocytes, the human non-activated and PWM-activated lymphocytes were also determined to have an augmented dThd-incorporation rate at low avarone/avarol concentrations (Fig. 2). Again, this enhancing effect on DNA synthesis was measured between 1 and 2 μM. Compared to murine lymphocytes the degree of stimulation of human lymphocytes with avarone/avarol was less pronounced and amounted for PWM-activated cells at 1.8 μM of avarone (avarol) 136% (126%) (control: 100%; P value vs. control ≤ 0.001).

DISCUSSION

The data reported in this contribution confirm earlier findings [3] showing that avarone and avarol cause mitotic arrest of lymphocytes *in vitro* due to an interference with the microtubule system. The main conclusions drawn from this paper are that (a) DNA synthesis of T lymphocytes and of B lymphocytes is differentially inhibited by both antimitotic drugs and (b) a dose-dependent stimulation of DNA synthesis by these drugs occurs only in B lymphocytes.

Avarone and avarol were found preferentially to inhibit DNA synthesis of T lymphocytes from both mouse spleen and human peripheral blood. The T lymphocytes from spleen were determined to be 2.3–2.5-fold more sensitively inhibited (statistically significant; P-value: ≤ 0.001) than the B lymphocytes. In the future we have to elucidate whether the observed antileukemic activity *in vivo* [4] is restricted to T-cells derived leukemias only.

In the last few years it has become clear that cytoskeletal proteins (actin and tubulin) are also regulator elements of gene expression in multicellular organisms (for review see: [12]). Disruption of microtubule architecture or deterioration of microtubule functions by microtubule poisons may result in modulation of gene expression, either in the catabolic [13] or anabolic direction [14]. Using Swiss 3T3 cells it was shown that colchicine enhances the rate of hormone-dependent DNA synthesis [14, 15]. The presented data ascribe avarone and avarol such an activity also. An interesting feature of the two antimitotic drugs, that might attain therapeutical importance, is their differential effect on cells. Only DNA synthesis in B lymphocytes from both mouse spleen (stimulated with the B cell mitogen LPS) and human peripheral blood (stimulated by the T and B cell mitogen PWM) was positively affected (P-value: ≤ 0.001) at low concentrations of avarone or avarol. The DNA synthesis in T lymphocytes was not stimulated by the drugs. It is not known which biochemical events, that are sensitive to the functional state of the microtubules, are involved in the restrictive control of DNA synthesis. Although microtubules are apparently not present in the nucleus, tubulin has been detected among the non-histone proteins [16]. Further experimental evidence suggests an interaction of microtubules with the nuclear pore-complex [17]. Thus, micro-

tubule formation under experimental conditions has been shown to occur from the pores towards the nuclear sap [18]. Results from studies with microtubule-disrupting drugs, e.g., colchicine [14], vinblastine [19] and avarone-avarol ([3] and this paper) suggest that an increase of the concentration of free tubulin stimulates mitotic growth. Based on our recent data we assume that changes in the array of microtubules and/or alterations of the concentration of free tubulin dimers may impair posttranscriptional control mechanisms at the level of nuclear-envelope nucleoside triphosphatase [20], essential for nuclear-cytoplasmic transport of poly(A) + mRNA [21].

The hitherto elucidated characteristics to be (a) preferentially antileukemic [4], (b) T-lymphocyte-specific and (c) anti-mutagenic [22], provide the cytostatic agent(s) avarone/avarol with promising properties utilizable for an application also in human cancer treatment.

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