Suppression of the Modulatory Effects of the Antileukemic and Anti-Human Immunodeficiency Virus Compound Avarol on Gene Expression by Tryptophan

Heinz C. Schröder, Rosemarie Wenger, Horst Gerner, Petra Reuter, Yoshiyuki Kuchino, Dusan Sladić, and Werner E. G. Müller

Institut für Physiologische Chemie [H. C. S., R. W., H. G., P. R., W. E. G. M.], Universität, Duesbergweg 6, 6500 Mainz, West Germany; Biophysics Division [Y. K.], National Cancer Center Research Institute, Tsukiji, Chuo-ku, Tokyo 104, Japan; Faculty of Science [D. S.], Department of Chemistry, Belgrade, Yugoslavia

ABSTRACT

The amino acid L-tryptophan is known to be a modulator of many processes of cell metabolism. In this contribution we show that L-tryptophan interferes with some biological effects of the antileukemic and anti-human immunodeficiency virus agent avarol, possibly by different mechanisms. Avarol has been shown to be able to modulate posttranscriptional events of mRNA synthesis, resulting in an increase of the base-sequence complexities of the nonabundant and rare mRNA classes. Here it is demonstrated that this change in mRNA abundance distribution is accompanied by an increase in the level of some specific, low abundant RNAs (ras and c-myc). Addition of L-tryptophan was found to abolish avarol-caused gene relaxation in L1210 mouse leukemia cells. In addition, L-tryptophan suppressed the induction of γ-interferon mRNA production in human peripheral blood lymphocytes. At the level of DNA, L-tryptophan inhibited the production of strand breaks by cytotoxic avarol concentrations in Friend erythroleukemia cells in vitro. Moreover, it competed with avarol for binding to the nuclear envelope binding site; this effect was not shown by other amino acids.

INTRODUCTION

The sesqueripenoid hydroquinone avarol and its quinone derivatived avarone are present in large quantities in the marine sponge Dysidea avara (1, 2). The isolated compounds have been shown to be potent antileukemic agents in vitro and in vivo (3). Avarol (avarone) displays a high selective cytotoxicity in L5178y mouse lymphoma cells with an ED50 of 0.93 μM (0.62 μM) (3) and in L1210 mouse leukemia cells with an ED50 of 1.08 μM (0.95 μM) (4), relative to all nonlymphoid cells tested (3). Normal murine and human blood lymphocytes are inhibited by both compounds with an ED50 of 2–6 μM (5). At lower concentrations (0.3 μM), avarol and avarone show a significant antiviral (anti-human immunodeficiency virus) activity in the human T-lymphotropic virus IIIb/H9 cell system (6), very likely by inhibition of HIV-induced expression of UAG suppressor glutamine tRNA (7). The cytostatic effect of avarol/avarone seems to be due to an inhibition of mitosis by an interference of the compound with the formation of microtubules (8). Further, both compounds inhibit SOD activity in vitro and in vivo (9, 10), suggesting that the cytotoxic effect of avarol and avarone is caused by an increase of the concentration of endogenously produced superoxide radicals. This assumption is in line with the finding that avarol inhibits predominantly growth of those cell lines which have low levels of SOD (11). The SOD seems to accelerate the conversion of avarol and avarone to a semiquinone intermediate (9). Moreover avarol itself is able to form superoxide radicals during its oxidation in vitro to the corresponding semiquinone radical in the presence of oxygen (9, 11). The formation of superoxide radicals by avarol, which can be converted to more reactive hydroxyl radicals, e.g., by Haber-Weiss reaction (12), has been shown to result in DNA damage, which occurs at cytotoxic drug concentrations both in vitro and in FLC; there are no hints for a formation of DNA-aavarol adducts (11).

One most notable property of avarol is its ability to alter the frequency distribution of mRNA in the low-abundance mRNA classes (4). It has been suggested that these changes in sequence complexity occur by modulation of posttranscriptional selection mechanisms during gene expression (4). However, they may also reflect alterations at the transcriptional level. Recently we found that avarol induces the expression of IFN-γ; the level of actin mRNA was not altered (13).

In this study, we investigated the influence of tryptophan on some avarol-caused effects which may be mechanistically coupled with the formation of oxygen radicals. However, during the course of this investigation it became also evident that the antagonistic effects of this amino acid may be due not only to its potential to act as hydroxyl radical scavenger, but also to its ability to interact with specific avarol binding sites within the cell.

MATERIALS AND METHODS

Materials. The materials were obtained as follows: [γ-32P]ATP (specific activity, 3 Ci/mmol), [γ-32P]GTP (10 Ci/mmol), [α-32P]dGTP (3000 Ci/mmol), [5-3H]dCTP (30 Ci/mmol), [5,6-3H]uridine (50 Ci/mmol), [3H]poly(U) (15 Ci/mmol), and L-[5-3H]tryptophan (30 Ci/mmol) from Amersham Buchler International (Buckinghamshire, England); DNA polymerase I (Escherichia coli; 11388 units/mg) and PMSF from Sigma Chemical Co. (St. Louis, MO); hydroxyapatite (DNA-grade Bio-Gel HTP) from Bio-Rad (Richmond, CA); glycerol, histidine-β, 12-O-tetradecanoylphorbol 13-acetate, and PMSF from Sigma Chemical Co. (St. Louis, MO); hydroxyapatite (DNA-grade Bio-Gel HTP) from Bio-Rad (Richmond, CA); oligo(dT)-cellulose from Collaborative Research (Waltham, MA); messner-activated paper from Medac (Hamburg, FRG); and nitrocellulose sheets (BA 85) from Schleicher & Schuell (Dassel, FRG).

Avarol was isolated from the sponge Dysidea avara by the method described previously (2, 3). [3H]Avarol was prepared by catalytic exchange with tritium-labeled water (14) and purified by high-pressure liquid chromatography (15); this material had a specific radioactivity of 24.2 Ci/mmol. For the experiments, avarol was dissolved in dimethyl sulfoxide at a concentration of 10 mg/ml; the final dimethyl sulfoxide concentration was maximally 0.5% and in the cell culture experiments,
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0.05%: at these concentrations dimethyl sulfoxide did not influence the reactions.

Cell Cultures. L1210 mouse leukemia cells (ATCC CCL 219) were grown in Eagle’s minimum essential medium supplemented with 1% horse serum (16). L5178y mouse lymphoma cells were cultivated and labeled with [5-3H]uridine as previously described (16, 17). Friend leukemic cells (FLC) were grown in Joklik minimal essential medium (without NaHCO3, but with N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid) supplemented with 10% fetal calf serum (11).

Stimulation of IFN-γ Production in Buff Coat Cells by Avarol. Human peripheral blood lymphocytes (buffy coat cells; obtained from the blood bank Bad Kreuznach) were isolated by Ficol-Hypaque density gradient centrifugation (18). After washing twice with RPMI 1640 medium, the cells were suspended at a density of 5 × 10^6 cells/ml in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% glutamine, and antibiotics (penicillin and streptomycin). Cultures (1 ml) were incubated for different time periods in the absence or presence of the indicated amounts of avarol and/or tryptophan at 37°C under humidified 5% CO2/95% air.

Isolation of Nuclei and Nuclear Envelopes. Nuclei were prepared from the cells according to the method described byBlobel and Potter (19), except that 1 mm PMMSF and 5 mm 2-mercaptoethanol were added to all the buffers used. The nuclear envelopes were isolated as described by Kaufmann et al. (20), and were stored at —70°C in buffer A (50 mm Tris-HCl, pH 7.4; 5 mm MgCl2, 5 mm 2-mercaptoethanol, 2.1 M sucrose, and 1 mm PMMSF).

RNA Preparations. All solutions had been supplemented with 0.05% diethylpyrocarbonate and autoclaved. L1210 cells (5 × 10^6 cells, incubated in the absence or presence of avarol with or without addition of tryptophan) were washed in culture medium, pelleted, and resuspended in 20 ml buffer B (10 mmTris-HCl, pH 8.5; 3 mm MgCl2, 10 mm NaCl) supplemented with 0.4 ml diethylpyrocarbonate and 0.5% Nonidet P40 (2°C). The nuclei of the detergent lysed cells were obtained by centrifugation (1000 × g; 4 min). Poly(A)+ RNA was isolated from the supernatant as follows. After centrifuging again (8000 × g; 10 min) the resulting postmitochondrial supernatant was applied onto a 15–40% sucrose gradient in buffer B. The polysomes obtained after centrifugation (21) were precipitated by addition of 2 volumes of 70% ethanol (—20°C; 4 h); yield: 65 A260 units from a 1-g cell pellet.

The extraction of the polysomal RNA was performed by the SDS/phenol/chloroform procedure (22).

Poly(A)+RNA was prepared by passage of polysomal RNA through an oligo(dT)-cellulose column equilibrated with 10 mm Tris-HCl buffer (pH 7.4, containing 400 mm NaCl and 0.2% SDS). 10 A260 units/ml of RNA were applied onto 1 g of oligo(dT)-cellulose. The elution of poly(A)+RNA from the column was done as described (21).

By hybridization with [3H]poly(U) (23) it was determined that the polysomal poly(A)+RNA preparation contained more than 97% of the poly(A)+RNA from the polysomes.

Hybridizations. cDNA was synthesized from polysomal poly(A)+RNA by reverse transcriptase from avian myeloblastosis virus, using [3H]dCTP (40 μCi/μmol) as radiolabeled precursor (24, 25). After incubation (37°C; 2 h) the cDNA was isolated by gel filtration on Sephadex G-50/Chelex 100. The synthesized [3H]cDNA appearing in the excluded volume was precipitated with ethanol and dissolved in distilled water at a concentration of 5 μg/ml (5 × 10^6 dpm/μg).

Hybridization of cDNA (2000 dpm) with 50 or 500 ng of RNA in 20-μl capillaries was performed essentially as described previously (4, 26). After boiling the sealed capillaries for 3 min and subsequent incubation at 70°C for the time required to reach the desired Rd value, the content of each capillary was mixed with 210 μl nuclease S1 buffer. Two aliquots were taken from this mixture and were either digested with S1 nuclease or incubated without this enzyme. The amount of cDNA hybridized was calculated from the ratio of the amounts of acid-precipitable radioactivity in both aliquots.

Analysis of Hybridization Kinetics. The base-sequence complexity of RNA has been shown to be proportional to the Rd value (= Rd value at 50% hybrid formation) (27, 28). The Rd values were obtained by plotting the percentage of cDNA hybridized versus log(Rd). From each value of cDNA counts in the graph, which was the average of duplicate determinations, 5% background was subtracted, representing cDNA which was resistant to S1 nuclelease digestion. The Rd values were corrected by multiplication with the proportion of the cDNA present in each frequency class (29). The complexity of the unknown RNA (Cs) was determined by comparison with the known complexity of globin mRNA (Cg) according to the equation Cg Cs = (Rdms,a)(Rdms,b) (29); molecular weight of globin mRNA, 4 × 10^6; Rdms,a, 6.6 × 10^-4 mol x s/1 (26). The number of different mRNA species and the concentration of individual mRNA molecules per cell were estimated using the following values (29, 30): average size of mRNA of L-cells, 6.8 × 10^3; mRNA content per L-cell, 0.18 pg (this value equals to 170,000 averaged-sized mRNA molecules).

In Vitro RNA Transport Measurements. Nucleocytoplasmic efflux of RNA was determined as described previously (17, 31), using isolated nuclei from L5178y cells that had been prelabeled with [3H]uridine. The nuclei were suspended in transport medium (17, 31) to a concentration of approximately 2.5 × 10^6/ml and preincubated at 22°C for 10 min in the absence or presence of avarol and/or tryptophan. The poly(A)+RNA released in the postnuclear supernatant after a 30-min incubation period was detected by binding to poly(U) paper (messenger-activated paper) and counting of bound radioactive activity (32).

Dot Blot Hybridization of RNA. Total RNA was isolated as described by Cathala et al. (33). The dot blot hybridization assay was performed as described by White and Bancroft (34) with the modifications given in (35). The baked nitrocellulose sheets were hybridized with the 32P-labeled probes as described by Maniatis et al. (36). As probes, we used human IFN-γ cDNA (39), cloned into the pUC vector, partial human c-myc cDNA (pRyc7.4) (37), the 46-base pair EcoRI fragment (clone BS-9) containing the ras oncogene of Harvey murine sarcoma virus (38) inserted into pBR322, and the 600-base pair 3’UTP fragment of the human β-actin gene (40) cloned into pBR322. The cDNA probes were labeled with [α-32P]dUTP by nick-translation (41) to a specific activity of 6–7 × 10^7 cpm/μg DNA. Exposition of the dried nitrocellulose to Kodak XAR-5 X-ray film (Eastman Kodak) with one intensifying screen was done for 2 days at —70°C.

Enzyme Preparations. Nuclear envelope NTPase was purified from rat liver to apparent homogeneity as described previously (42). Protein kinase C was isolated from rat brain and purified by binding to human erythrocyte inside-out vesicles, followed by chromatography on phenyl-Sepharose, as described by Wolf et al. (43).

Enzyme Assays. Nuclear envelope NTPase activity was determined, essentially as described previously (42), in the absence or presence of 20 μM poly(A); at this poly(A) concentration the NTPase is stimulated about half-maximally (31).

The reaction mixture to measure nuclear envelope-associated NII-like protein kinase activity (44) contained, in a final volume of 100 μl, 50-μg envelope protein; GTP was used to measure only NII-like kinase activity, which utilizes both ATP and GTP, in contrast to kinase N1 (45). After incubation for 0–30 min at 22°C, 32P, incorporated into protein was determined by trichloroacetic acid precipitation or electrophoresis followed by autoradiography (see below). In some assays, reaction mixtures were supplemented with 2.5 mg/ml of phosvitin as exogenous substrate.

Protein kinase C activity was determined as described previously with or without 1 mg/ml of histone III-S as exogenous substrate (44). For measurement of phosphorylation of nuclear envelope protein by exogenous protein kinase C, 1.1 units/ml of purified rat brain kinase C were added to 50-μg envelope protein in reaction mixture supplemented with 0.02 μg/ml of 12- O-tetradecanoylphorbol-13-acetate. Incubations were performed for 0–10 min at 30°C and incorporation of 32P into protein was determined as described above.

Binding of Avarol and Tryptophan to Nuclear Envelopes. Nuclear envelopes from L5178y cells were suspended in buffer A at a final concentration of 0.17 mg/ml of protein (final volume, 100 μl). After addition of increasing concentrations of [3H]a varol or [3H]tryptophan (0.001–1 μM) without or with radiolabeled avarol or tryptophan or other amino acids (100 μM), the suspensions were incubated at 22°C for 20 min. The nuclear envelopes were then pelleted by centrifugation (11,000 × g; 10 min; 4°C), washed with buffer A and bound and unbound
radioactivity were determined. The data were analyzed by the mathematical Ligand program (46).

Measurement of DNA Strand Breakage in FLC. DNA damage was measured by fluorimetric analysis of the rate of DNA unwinding under alkaline conditions (47). Cultures of FLC (initial concentration, 1.75 × 10^7 cells/35 ml) were incubated for 0–24 h in the absence or presence of different concentrations of avarol with or without tryptophan. After cooling in ice water, the cells were pelleted by centrifugation, washed, and aliquoted (10 tubes). After lysis of the cells with an urea/detergent mixture, an alkaline solution was added, and DNA strand unwinding was allowed to occur at 22°C for 60 min. The samples were then neutralized, and the amount of residual double-stranded DNA was estimated by using ethidium bromide as fluorescence dye. The number of strand breaks per cell (given in Qo units; Ref. 48) was calculated from the measured rate of DNA unwinding by comparing with the effect produced by γ-rays. 1 Qo unit equals to about 100 strand breaks per cell.

Analytical Methods. The incorporation of [32P]phosphate into nuclear envelope polyepitides was determined after incubating L5178y envelopes with [γ-32P]ATP or [γ-32P]GTP under the conditions outlined under protein kinase NII and C assay. The reactions were terminated by adding ice cold 15% trichloroacetic acid. The pelletted proteins were boiled in sample buffer and then electrophoresed in 10% polyacrylamide slab gels in the presence of 0.1% SDS, using the system described by Laemmli (49). Autoradiography of the dried gels was for 2 days at −70°C.

Protein was estimated according to the method of Lowry et al. (50) using bovine serum albumin as a standard. The concentration of RNA was measured spectrophotometrically (1 OD260 = 37.1 μg/ml of RNA). The poly(A) content was determined by titration with [3H]poly(U) (23, 27).

RESULTS

Effect of Tryptophan on Avarol-caused Changes of RNA Sequence Complexity. Previously we demonstrated (4) that treatment of L1210 mouse leukemia cells with avarol (at ED₉₀ concentration) causes significant changes in the frequency distribution of messenger sequences in the nonabundant and rare mRNA classes. Using the same cell system, we investigated now whether this effect of avarol can be influenced by L-tryptophan. The reason for this was the fact that tryptophan can act as a scavenger of hydroxyl radicals, which may be formed from superoxide radicals produced by avarol in the presence of oxygen; free radicals are assumed to be implicated in cellular differentiation processes and in derepression of genes (51).

As we had already reported, treatment of L1210 cells with the ED₉₀ concentration of avarol (≈1.1 μM) for 72 h did not significantly change the cellular content of polysomal poly(A)^+RNA (~0.18 pg/cell) and of total nuclear RNA (~1.4 pg/cell). The same values were found in cells incubated with avarol in the presence of 1 mM L-tryptophan (Table 1). Both additives did not alter also the poly(A) content of both RNA fractions (Table 1).

Results of experiments showing the influence of avarol treatment (ED₉₀ concentration) of L1210 cells for 72 h on the hybridization kinetics of the polysomal poly(A)^+RNA with cDNA, transcribed from polysomal poly(A)^+RNA of untreated cells, have been published (4). Now we determined, in the same set of experiments, the influence of L-tryptophan, if added to the cells at a concentration of 1 mM together with avarol, on the drug-caused modulation of gene expression. The results showing the sequence complexity and relative abundance of the poly(A)^+RNA in the three frequency classes 1 (abundant), II (nonabundant), and III (rare), derived from the complex hybridization kinetics, are summarized in Table 2. The results revealed that coaddition of 1 mM L-tryptophan with avarol (ED₉₀ concentration) to the cells strongly suppressed the avarol-caused increase in sequence complexity of the nonabundant (II) and rare (III) mRNA classes. Addition of avarol alone increased the complexity in class II from 324 (329) (untreated) to 411 (421) (treated) cells different mRNA species and in class III, from 1,765 (1,703) to 2,090 (2,141). In the simultaneous presence of L-tryptophan, however, 355 (344) different mRNA species were calculated for class II, and 1,861 (1,779) for class III (Table 2). The number of different species of class I messengers did not change in the presence of avarol (and tryptophan). In contrast to the diversity of mRNA species, which increased in the presence of avarol, the copy number of individual mRNA species decreased in drug-treated cells; this effect could also be suppressed by coaddition of tryptophan. Tryptophan itself displayed no effect on mRNA abundance distribution.

Effect of Tryptophan on Avarol-caused Induction of IFN-γ mRNA Production. Avarol has been shown to be able to cause an induction of IFN-γ gene in vitro in buffy coat cells (human peripheral blood lymphocytes). Induction of IFN-γ production was found to be maximal after incubation of the cells in the presence of 0.75 μg/ml (=2.4 μM) of avarol; at higher concentrations [1.5 μg/ml; corresponding to the ED₉₀ for [3H]thymidine incorporation into DNA (5)] the production of IFN-γ was inhibited (13). As shown in Fig. 1, the IFN-γ mRNA levels paralleled the IFN-γ production. Fig. 1 additionally shows that L1210 mouse leukemia cells, used to study the avarol-caused effect on mRNA abundance distribution, contain ras and c-myc mRNA. The formation of the ras and c-myc gene transcripts increased after incubation of the cells with avarol, reaching maximal levels in the presence of the ED₉₀ concentration of this compound; thereafter the concentration of both mRNAs decreased (Fig. 1). Thus the production of ras and c-myc mRNA displays different changes in response to avarol as compared to IFN-γ mRNA, and seems to be linked with the cytotoxic effect of this compound. The mechanism of the avarol-caused induction of IFN-γ mRNA production is not known, but it could be related to the mechanism(s) underlying the gene expression modulatory action of avarol. Therefore, we determined if coaddition of L-tryptophan together with avarol inhibits the production of IFN-γ mRNA. The IFN-γ mRNA was detected by RIA dot-blot hybridization using cloned human IFN-γ cDNA. Quantitation of the IFN-γ mRNA was performed by densitometric scanning of the autoradiograms using a standard curve. As shown in Fig. 2A, buffy coat cells cultivated in the absence of avarol contained no IFN-γ transcripts. Addition of avarol at optimal concentration (=0.75 μg/ml or 2.4 μM)
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Table 2 Frequency distribution of polysomal poly(A)* RNA from L1210 cells after treatment without or with avarol in the absence or presence of L-tryptophan

Cells were incubated for 72 h in medium, supplemented with 1.1 µM avarol or 1.1 µM avarol plus 1 mM L-tryptophan or 1 mM L-tryptophan, or without additives, and polysomal poly(A)* RNA was isolated and analyzed by hybridization to cDNA transcribed from polysomal poly(A)* RNA of untreated cells. The frequency classes are: I, abundant; II, nonabundant; and III, rare. Calculations were performed as described under "Materials and Methods." Data from two independent experiments are given. Data for untreated and avarol-treated cells have partly been published previously (4).

<table>
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<th>Frequency class</th>
<th>Exp. no.</th>
<th>Proportion of cDNA hybridized (%)</th>
<th>Observed $R_b$ (M × s)</th>
<th>Corrected $R_b$ (M × s)</th>
<th>Total complexity (daltons)</th>
<th>Different mRNA species</th>
<th>Copies of mRNA species per cell</th>
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µM) resulted in an induction of production of IFN-γ mRNA, which reaches maximal levels after an incubation period of 5 days (data not shown; see also Ref. 13). Coaddition of L-tryptophan significantly suppressed the induction of IFN-γ gene transcripts; in the presence of 0.1 mM tryptophan, the maximal concentration of IFN-γ mRNA was only about 25% of that measured without added amino acid, and in the presence of 1 mM tryptophan, less than 5% (Fig. 2A). As a reference gene, we used the β-actin gene; the expression of this gene was not altered in the presence of avarol alone or together with tryptophan (Fig. 2B; see also Ref. 13).

Competition of Tryptophan and Avarol for Nuclear Envelope Binding Site. Tryptophan is known to be an amino acid which is able to modulate gene expression, particularly at the level of nucleocytoplasmic mRNA transport (52). Administration of tryptophan to fasted rats has been shown to result in an enhanced efflux of mRNA from isolated liver nuclei and an enhanced nuclear envelope nucleoside triphosphatase (NTPase) activity (53); this enzyme is thought to mediate the translocation of mRNA through the nuclear envelope pore complexes (52). Tryptophan has been shown to bind to isolated nuclei and nuclear envelopes (54). In a previous study we demonstrated that a significant amount of [3H]a varol fed to rats 0.5 or 12 h before killing accumulated in the nuclear envelope-containing fraction of liver cells (11). Now we determined that tritium-labeled avarol strongly binds to isolated nuclear envelopes from L5178y cells. Binding experiments revealed that the L-cell envelopes contain two classes of avarol binding sites, as determined by Scatchard analysis, using the Ligand program (46) (Fig. 3A). The association constant ($K_a$) for the high-affinity binding site was found to be $4.0 \times 10^8$/M and that for the low-affinity site, $1.3 \times 10^8$/M. The numbers of binding sites were determined to be $4.9 \times 10^{13}$ per mg of nuclear envelope protein (corresponding to $7.9 \times 10^4$ sites per nuclear envelope; high-affinity sites) and $6.9 \times 10^{14}$ per mg of nuclear envelope protein (corresponding to $1.1 \times 10^6$ sites per nuclear envelope; low-affinity binding sites). These numbers of binding sites were found to be in the same order of magnitude as those for [3H]-tryptophan (Fig. 3B). Using this amino acid, we determined for L-cell envelopes two populations of binding sites, too, with $K_a$ values of $2.9 \times 10^9$/M and $7.1 \times 10^9$/M, respectively; the numbers of binding sites were $3.9 \times 10^{13}$/mg = ($6.2 \times 10^4$/ nuclear envelope) and $5.1 \times 10^{14}$/mg = ($8.2 \times 10^6$/ nuclear envelope), respectively. To establish whether the binding sites for avarol and tryptophan are related or identical, competition experiments were performed. The results revealed (Table 3) that addition of excess tryptophan (0.1 mM) was able to prevent binding of [3H]a varol to the L-cell envelopes. This effect was not shown by all others of the 20 amino acids commonly found in proteins (Table 3).

Next we determined whether binding of avarol to the nuclear envelope influences efflux of mRNA out of the nucleus and nuclear envelope NTPase and phosphoprotein kinase activities; the latter enzyme has been suggested to be involved in down-regulation of NTPase activity and mRNA efflux rate by enhancing the poly(A) binding affinity of the mRNA carrier in the envelope (52). However, neither the amount of mRNA released from isolated L-cell nuclei nor the nuclear envelope NTPase and NII-like kinase activities were found to be altered in the presence of different avarol concentrations (1–100 µM); Table 4. The same result was found using the purified, homogeneous NTPase from rat liver (not shown). Addition of 0.1 to 1 mM tryptophan to isolated L-cell nuclei and nuclear envelopes also did not affect the mRNA translocation system, in contrast
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Fig. 1. Alterations of IFN-γ, ras and c-myc mRNA levels in cells treated with different concentrations of avarol. Buffy coat cells (used to determine IFN-γ mRNA, whose production is inducible by avarol in these cells; see Ref. 13) and L1210 cells (measurement of ras and c-myc mRNA levels) were incubated for 72 h in the presence of different ED₅₀ concentrations of avarol. Total RNA was then extracted, spotted onto nitrocellulose (6 µg RNA per dot) and hybridized with the ³²P-labeled IFN-γ, ras and c-myc DNA probes. A, dot-blot detection of RNAs after a 72-h incubation of cells in the absence (1) or the presence of the respective 1/8 x ED₅₀ (2), 1/4 x ED₅₀ (3), 1/2 x ED₅₀ (4), ED₅₀ (5), and 2 x ED₅₀ (6) concentrations. Autoradiograms were scanned and the relative absorbances (in arbitrary units) were calculated using a standard curve constructed from dot-blots obtained after serial dilutions of total RNA. For the results shown in (B) four independent experiments were evaluated. (●) IFN-γ mRNA; (○) ras mRNA; (△) c-myc mRNA.

Fig. 2. Suppression of avarol-induced production of IFN-γ transcripts in human peripheral blood lymphocytes by tryptophan. Total RNA was isolated from cells that had been incubated for 72 h in the absence (lane a) or presence of 0.75 µg/ml of avarol (lane b) without (top) or with addition of 0.1 mM tryptophan (bottom), and 5 µg RNA each were analyzed by dot-blot hybridization using either ³²P-labeled cloned IFN-γ (A) or β-actin cDNA probes (B).

Fig. 3. Scatchard plots of [³H]avarol and [³H]tryptophan binding data. The Ligand-computed best fit curve (—) as well as the resolved high-affinity (---) and low-affinity (-- --) binding sites are given. A, avarol; B, tryptophan.

Table 3 Competition of several amino acids with [³H]avarol binding to nuclear envelopes from L5178y cells

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Bound [³H]avarol (%)</th>
<th>Amino acid</th>
<th>Bound [³H]avarol (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gly</td>
<td>98</td>
<td>Tyr</td>
<td>102</td>
</tr>
<tr>
<td>Ala</td>
<td>101</td>
<td>Trp</td>
<td>13</td>
</tr>
<tr>
<td>Val</td>
<td>102</td>
<td>Pro</td>
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</tr>
<tr>
<td>Met</td>
<td>102</td>
<td>His</td>
<td>101</td>
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Shown in Fig. 4B, avarol at a concentration of 100 µM had also no influence on the phosphorylation pattern of L-cell envelopes, obtained after incubation with endogenous NII-like protein kinase.

Recently we demonstrated that purified protein kinase C reversibly binds to isolated rat liver nuclei (44). Binding of protein kinase C resulted in an inhibition of the ATP-dependent mRNA efflux and in a decrease of NTPase activity (44). Therefore, we investigated if avarol could affect the kinase C-mediated incorporation of phosphate in nuclear envelope protein (from L5178y cells) and nuclear envelope functions related to mRNA transport. However, avarol (100 µM) was found to influence neither the resulting nuclear envelope phosphorylation pattern (Fig. 4C) nor NTPase-mediated mRNA transport in vitro (data not shown).

Prevention of Avarol-caused DNA Damage by Tryptophan. Avarol has been shown to produce superoxide ion radical in solution (11). One consequence of the generation of superoxide radicals and hydroxyl radicals, which may arise from the reaction of superoxide radicals with hydrogen peroxide [Haber-
Avarol has been shown to be provided with the unique ability to alter the frequency distribution of messenger sequences within the polysomal mRNA and the nuclear RNA (4). This avarol-caused modulatory effect on gene expression might be partially due to the induction of genes not expressed in untreated cells, such as IFN-γ gene (13). Now we found that the avarol-caused increase in the degree of complexity of mRNA species in the nonabundant messenger class could be suppressed by coaddition of tryptophan. The same amino acid prevented also the induction of IFN-γ mRNA production by avarol in vitro in buffy coat cells. The mechanism(s) by which avarol causes these obviously specific effects on gene expression is (are) not known. One possibility may be that these processes are triggered by active oxygen species which may be formed in the presence of avarol. It has been suggested that free radicals are involved in cellular differentiation processes and in depression of genes (51). Avarol has been shown to be able to produce superoxide radicals during its conversion to the semi-quinone derivative in the presence of dioxygen (11); these superoxide radicals may be intracellularly converted to more reactive hydroxyl radicals (12). Tryptophan, on the other hand, has been shown to be a potent scavenger for hydroxyl radicals (56), and hence could abolish effects of avarol mediated by free radicals.

There is evidence, however, that abolition of avarol-caused effects by tryptophan may occur also through some other mechanism. Thus, avarol and tryptophan were found to compete for obviously the same binding site on L5178y mouse lymphoma cell nuclear envelopes. In a previous study we had already demonstrated autoradiographically that [3H]avarol accumulates in the cytoplasm of NIH-3T3 cells and lymphocytes close to

**DISCUSSION**

Avarol has been shown to be provided with the unique ability to alter the frequency distribution of messenger sequences within the polysomal mRNA and the nuclear RNA (4). This avarol-caused modulatory effect on gene expression might be partially due to the induction of genes not expressed in untreated cells, such as IFN-γ gene (13). Now we found that the avarol-caused increase in the degree of complexity of mRNA species in the nonabundant messenger class could be suppressed by coaddition of tryptophan. The same amino acid prevented also the induction of IFN-γ mRNA production by avarol in vitro in buffy coat cells. The mechanism(s) by which avarol causes these obviously specific effects on gene expression is (are) not known. One possibility may be that these processes are triggered by active oxygen species which may be formed in the presence of avarol. It has been suggested that free radicals are involved in cellular differentiation processes and in depression of genes (51). Avarol has been shown to be able to produce superoxide radicals during its conversion to the semi-quinone derivative in the presence of dioxygen (11); these superoxide radicals may be intracellularly converted to more reactive hydroxyl radicals (12). Tryptophan, on the other hand, has been shown to be a potent scavenger for hydroxyl radicals (56), and hence could abolish effects of avarol mediated by free radicals.

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...the nucleus (57). Fractionation of liver cells of rats fed with [1H]avarol revealed that a significant amount of avarol was bound to the nuclear envelope (11). Based on reports showing tryptophan to bind to isolated nuclei and nuclear envelopes (54) and to modulate, if administered in vivo, nuclear envelope NTPase and phosphoprotein kinase activities (55) as well as mRNA translocation rate (53), we investigated the ability of nuclear envelopes from L5178y cells to bind avarol (in the absence and in the presence of tryptophan) and the effect of bound avarol on nuclear envelope enzyme activities involved in mRNA translocation. Our studies revealed the existence of two classes of binding sites for avarol in L-cell envelopes. Interestingly, the number of high affinity ($K_a \sim 10^9$/m) binding sites ($\sim 8 \times 10^4$ sites/nuclear envelope) was in the same order of magnitude as that determined for tryptophan ($\sim 6 \times 10^4$ sites/nuclear envelope). Most important was the finding that addition of tryptophan prevented the binding of avarol to the envelopes; this effect was not observed with other amino acids, indicating the tryptophan effect to be specific.

Alteration of gene expression, however, could also be a consequence of the production of DNA strand breaks by avarol, which might stimulate poly(ADP-ribosylation) of DNA binding proteins involved in gene regulation. Based on our knowledge that tryptophan is a strong radical scavenger (56), we tested whether DNA damage at cytotoxic avarol concentrations is inhibited by tryptophan. Using the technique of fluorometric analysis of DNA unwinding, tryptophan was found to suppress significantly the avarol-caused production of DNA strand breaks in FLC, very likely by scavenging of hydroxyl radicals derived from superoxide radical anions, which are formed in the presence of avarol and dioxygen. This assumption is supported by results showing avarol-caused DNA damage to be partially prevented also by coaddition of α-tocopherol, butylated hydroxyanisole, or butylated hydroxytoluene.

Changes in sequence complexities of mRNA might also be caused by altered nucleocytoplasmic mRNA transport. Recently we demonstrated that the restriction of immature mRNA to the nucleus is impaired in the presence of superoxide radicals generated in vitro by a superoxide radical-producing system (57, 58). This effect seems to be due to a disintegration of the complex formed between immature mRNA and nuclear matrix (58). Here we demonstrated that avarol binding to the envelope influenced neither mRNA translocation nor enzyme systems (NTPase, protein kinases NII and C) involved in regulation of this process. However, it cannot be excluded that avarol affects nucleocytoplasmic mRNA transport more indirectly by depletion of cellular ATP pool due to activation of poly(ADP-ribose) polymerase (as consequence of DNA damage), which consumes NAD+ needed as a cofactor for ATP production.

Future studies must show if nuclear envelope-bound avarol interferes with oxidoreductases, in particular with NADH- and NADPH-cytochrome P-450 reductase activities found to be present in the nuclear envelope, e.g., from rat liver (59). Interestingly, much higher activities of these enzymes have been measured in the envelopes of hepatoma nuclei (60). The formation of free radicals in isolated nuclei in the presence of oxygen and NADPH has also been shown with some other quinone (or quinone-imine) antibiotics, e.g., daunorubicin, mitomycin C, actinomycin D, streptonigrin, and Adriamycin. The production of superoxide radicals (and possibly hydroxy radicals which can be scavenged by tryptophan) by avarol might be important for its antitumor activity, since tumor cells have been shown to contain reduced levels of SOD activity (61). From our results, a negative correlation between the sensitivity of a cell line to avarol and the respective cellular SOD level seems to exist (11). Further we have to clarify the nature of the nuclear envelope avarol binding site, which might be important not only for the action of avarol but also for the regulatory effects of the amino acid tryptophan on gene expression.

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