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Induction of γ-Interferon by Avarol in Human Peripheral Blood Lymphocytes

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Avarol is a cytostatic and anti-human immunodeficiency virus (HIV) agent. In this study, the avarol caused induction of γ-interferon (IFN-γ) in buffy coat cells (human peripheral blood lymphocytes) is demonstrated by immunological and molecular biological techniques. IFN-γ production was detected after a 24-hr incubation period with avarol; maximal production was obtained after 5 days in the presence of the optimal avarol concentration of 0.75 μg/ml. Blotting experiments using human IFN-γ cDNA and β-actin cDNA containing plasmids showed that in the absence of avarol no IFN-γ transcripts were present in lymphocytes. Already after a 24-hr incubation with avarol, IFN-γ gene induction was detected, and maximal induction was found after a 5-day incubation period. The enhanced IFN-γ production seems to be caused by a change at the transcriptional and/or post-transcriptional level, but not during subsequent nucleocytoplasmic transport of mRNA. This molecular event is specific, at least in relation to the expression of the β-actin gene. Our studies demonstrate that avarol displays, besides its potential anti-tumor and anti-HIV activity, a potential immunomodulating effect.

Key words: Avarol — Interferon-gamma

The interferons appear to be of central importance in immune regulation and are produced by different body cells in response to stimulation by macromolecules, viruses, bacteria and foreign cells.[1] They are classified as α-interferon (produced by null lymphocytes, B-lymphocytes and macrophages), β-interferon (produced by fibroblasts, myoblasts and lymphoblasts) and γ-interferon (IFN-γ[*4]) (produced by T-lymphocytes).[2, 3] Four main enzyme systems have been implicated in the overall antiviral action of interferon: (a) pppA2′p5′A2′p5′A synthetase,[4] (b) (2′-5′)-oligoadenylate-dependent endoribonuclease,[4] (c) the poly(A)-specific 2′,3′-exoribonuclease[5–7] and (d) a specific protein kinase.[8] The IFN-α, β, and γ induce an antiviral state against single-stranded infectious RNA viruses (e.g., encephalomyocarditis virus), double-stranded RNA viruses (e.g., reovirus), DNA-containing viruses (e.g., simian virus[40]) and retroviruses.[9] The antiviral spectrum of IFNs against retroviruses includes both animal retroviruses (e.g., mouse mammary tumor virus[10]) and human retrovirus (HIV[11, 12]).

Recently we reported that the sesquiterpenoid hydroquinone avarol, a cytostatic agent inhibiting the growth of T cell lymphoma lines,[13, 14] effectively inhibits HIV replication *in vitro* in the HTLV-IIIB/H9 cell system.[15] The molecular basis of the anti-HIV activity of avarol is not known. The compound does not inhibit mammalian DNA polymerases α, β and γ, mammalian RNA polymerases I, II and III, or reverse transcriptase (from avian myeloblastosis virus and from HIV) (unpublished). Since IFNs are known to suppress the replication of retroviruses (see above), we studied the possibility that avarol acts as an IFN-γ inducer. We show here that avarol causes IFN-γ gene induction *in vitro* in peripheral human blood lymphocytes. The avarol-caused induction is not an unspecific effect at the level of tran-

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[*4] The abbreviations used are: AIDS, acquired immunodeficiency syndrome; EDTA, ethylenediaminetetraacetic acid; HIV, human immunodeficiency virus; HTLV, human T-cell leukemia/lymphotropic virus; IFN-γ, γ-interferon; IL-2, interleukin 2.

scription or post-transcription. On the contrary, the compound induces IFN-γ gene expression without altering the level of actin transcripts. We have selected the cytoskeletal β-actin gene as a reference gene, because it codes for one of the most abundant cellular proteins.[16]

Materials and Methods

Materials Oligo(dT)-cellulose was obtained from Collaborative Research, Waltham, MA (USA); [α-^{32}P]dTTP (3,000 Ci/mmol) from the Radiochemical Centre, Amersham (England); RNasin, phosphoenolpyruvate and pyruvate kinase from Boehringer Mannheim, Mannheim (Germany); purified recombinant human interferon γ and anti-human interferon γ (monoclonal antibody, peroxidase-conjugated[17]) from Hoffmann-La Roche, Basel (Switzerland).

Avarol was isolated from *Dysidea avara*,[18] which was collected in the Bay of Kotor (Yugoslavia). Avarol was dissolved in 0.05% dimethyl sulfoxide (final concentration); this solvent had no influence on cell growth or IFN-γ induction.

Treatment of Human Peripheral Blood Lymphocytes with Avarol and Stimulation of IFN-γ Production Buffy coat cells (human peripheral blood lymphocytes) from the Bad Kreuznach blood bank were isolated by Ficoll-Hypaque density gradient centrifugation[19] and subsequently washed twice with RPMI 1640 medium. The lymphocytes were suspended at a density of 5×10^6 cells/ml in RPMI 1640 medium containing 10% fetal calf serum, supplemented with 1% glutamine and antibiotics (penicillin and streptomycin). Cultures (1 ml) were incubated at 37° under humidified 5% CO_2/95% air for 0–96 hr, then centrifuged. The supernatant was collected and the IFN-γ titer was determined.

Interferon Assay IFN-γ was assayed by enzyme-immunoassay as described.[17] Briefly, 96-well flat-bottomed plates (Greiner, Nürtingen; Germany) were coated with anti-human IFN-γ. This antibody preparation did not cross react with IFN-α or IFN-β. Culture supernatants were then added at 50 μl per well together with 50 μl of peroxidase-labeled anti-human IFN-γ. After 24 hr at 4° the wells were washed with 3% ovalbumin in phosphate-buffered saline, containing 0.5% Tween 20. Finally, the peroxidase reaction was performed and the absorbance at 492 nm was determined essentially as described.[20] The calibration curve for the quantitation of the IFN-γ titer in the supernatants was obtained by using standard IFN-γ; the activity is expressed as IFN-γ NIH-units/ml of culture supernatant. The curve was linear in the range of 0–1,000 NIH-units/ml.

Recombinant DNAs As a specific human β-actin probe, we used the 600-bp 3′-UTP fragment cloned in pBR322. This fragment hybridizes specifically to mRNA coding for β-actin, but not to mRNA which codes for γ-actin or muscle-specific α-actin.[21,22] The human IFN-γ cDNA,[23] cloned into the pUC vector, was a gift of Dr. Jan Mous (Hoffmann-La Roche). The cDNA probes were nick-translated with [α-^{32}P]dTTP to a specific radioactivity of $6–7\times10^7$ cpm/μg DNA.[24]

Northern Blot Preparation Total RNA from lymphocytes was isolated according to the guanidinium thiocyanate extraction procedure of Chirgwin *et al.*[25] The purified total RNA was denatured at 56° for 30 min in electrophoresis buffer (40m*M* morpholinopropanesulfonic acid, 10m*M* sodium acetate, 1m*M* EDTA; pH 7.2) containing 50% dimethyl sulfoxide and 6% formaldehyde[26] and subjected to electrophoresis on 1.1% agarose (standard low-m_r; Bio-Rad Laboratories) gels containing 6% formaldehyde.[27] The separated RNAs were blot-transferred to nitrocellulose (BA 85; Schleicher & Schuell) and hybridized with the ^{32}P-labeled probes according to Maniatis *et al.*[27] under the conditions described earlier.[28] The dry nitrocellulose filters were exposed to Kodak XAR-5 X-ray film (Eastman Kodak) backed by one intensifying screen at −70° for 4 days.

Poly(A)$^+$ RNA was isolated from total RNA by oligo(dT)-cellulose chromatography.[27]

The concentration of the extracted RNA was calculated based on 1 OD_{260}=37.1 μg RNA/ml.

Dot Blot Analysis The dot blot hybridization assay was performed according to the method of White and Bancroft[29] with modifications.[30] Briefly, the RNA in the postnuclear supernatant was denatured by adding 7.5% formaldehyde and 6×SSC buffer,[27] and heating to 56° for 20 min. Aliquots obtained after adjusting the sample to 13×SSC were applied to nitrocellulose sheets. The baked sheets were hybridized with the ^{32}P-labeled IFN-γ-specific probe in the absence or presence of the ^{32}P-labeled actin cDNA probe as described by Maniatis *et al.*[27] The filters were exposed to X-ray films; the spots on the films were quantitated by scanning densitometry.

RNA Efflux from Isolated Nuclei Nuclei were isolated from buffy coat cells by the method of Blobel and Potter.[31] Then 7×10^6 nuclei were washed once in transport medium (50m*M* Tris-HCl [pH 7.6], 25m*M* KCl, 250m*M* sucrose, 2.5 m*M* $MgCl_2$, 0.3m*M* $MnCl_2$, 0.5m*M* $CaCl_2$, 5m*M* 2-mercaptoethanol, 5m*M* spermidine-HCl, and 10^3 units/ml of RNasin) and transferred into the transport medium supplemented with 2.5m*M* ATP, 5 m*M* Na_2HPO_4, 5m*M* phosphoenolpyruvate, and 35 units/ml of pyruvate kinase. The RNA efflux reac-

tions were carried out at 37° for 30 min. The reactions were stopped by cooling in ice-water and pelleting the nuclei by centrifugation (1,500*g*, 10 min, 4°). The RNA released in the postnuclear supernatant was analyzed by dot blot hybridization or by hybridization of Northern blots of this RNA to an IFN-γ-specific probe in the absence or presence of an actin-specific DNA probe. The details of the procedures to determine RNA efflux have been described.[32-34]

Statistical Evaluation Student's *t* test was employed to determine the significance of the differences in IFN-γ production.[35]

Results

IFN-γ Production by Lymphocytes after Avarol Incubation In the absence of avarol, buffy coat cells (human peripheral blood lymphocytes) produce very little IFN-γ (3±2 to 5±2 units/ml; Fig. 1). After addition of 0.75 μg/ml of avarol (=2.4μ*M*) a time-dependent increase of IFN-γ production was observed; maximal levels were determined after an incubation period of 5 days (82±11 units/ml; Fig. 1). The difference between the IFN-γ levels in the avarol-treated cultures and the controls was significant after an incubation period of 1 day (*P* value versus control; <0.005).

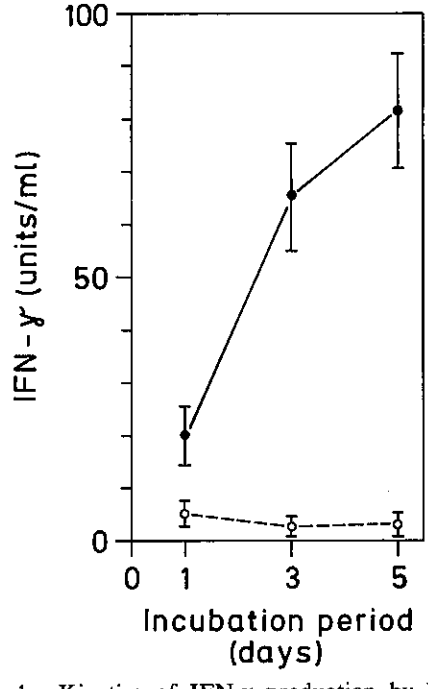

Fig. 1. Kinetics of IFN-γ production by buffy coat cells in the presence of 0 (○) or 0.75 μg/ml of avarol (●). The cells were incubated in the standard assay for 1, 3 or 5 days; then the supernatants were analyzed for IFN-γ content. Means (±SD) of 7 parallel experiments are given.

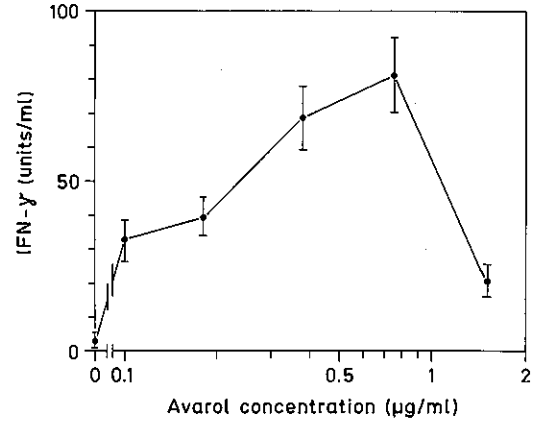

Fig. 2. IFN-γ production of buffy coat cells as a function of avarol concentration. The cells were incubated in the standard assay (5 days) in the presence of various avarol concentrations. The supernatants were analyzed for IFN-γ content; means (±SD) of 7 parallel experiments are given.

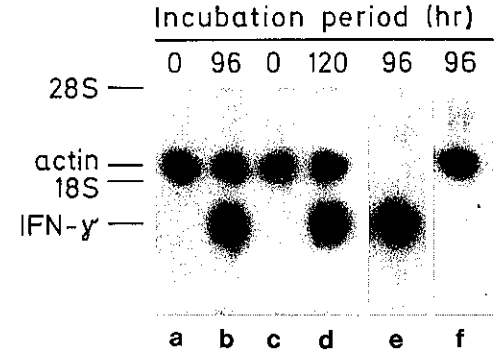

Fig. 3. Northern blot analysis of IFN-γ and actin transcripts after activation of human lymphocytes with avarol (0.75 μg/ml). At the indicated time after activation, the cells were harvested, and total RNA was isolated and assayed with ^{32}P-labeled actin- and IFN-γ probes. Lanes a–d, addition of actin cDNA probe together with IFN-γ probe to the hybridization assay; lane e, addition of IFN-γ probe and lane f, addition of actin probe. 18S and 28S indicate the positions of ribosomal RNA markers.

The extent of IFN-γ production by lymphocytes in the presence of avarol was dose-dependent (Fig. 2). A significant production was observed at a concentration of 0.1 μg/ml ($=0.32\mu M$; $P<0.005$). The maximal levels were measured in the presence of 0.75 μg/ml (82 ± 11 units/ml). At higher doses the IFN-γ production was inhibited. This effect presumably contributes to the cytotoxic effect of the compound; in a previous study[14] it was found that at a concentration of 1.5 μg/ml the incorporation rate of [^{3}H]dThd into DNA was inhibited by 50%.

IFN-γ Gene Expression in Avarol-treated Cells Transcription of IFN-γ gene and its inducibility by avarol in buffy coat cells were analyzed by RNA blot hybridization. Figure 3 shows the absence of the IFN-γ transcripts in untreated cells (lanes a and c); only the actin transcripts could be detected. However, after an incubation period of 96 hr or 120 hr in the presence of 0.75 μg/ml of avarol the IFN-γ transcripts were clearly detectable. The data presented in Fig. 3 indicate that the relative amount of the actin transcripts in lymphocytes remained unchanged, irrespective of the presence of avarol in the culture medium. In control experiments, using only one cDNA probe in the hybridization assay, the migration behavior of the IFN-γ(Fig. 3; lane e) or of the actin transcripts (lane f) from avarol-activated cells was determined.

To obtain an assessment of the dose-dependent appearance of IFN-γ transcripts a dot blot analysis was performed (Fig. 4). Cells treated with 0 μg/ml avarol were apparently free of IFN-γ transcripts (lane a), while cells treated with 0.1 (lane b) or 0.75 μg/ml avarol (lane c) contained significant amounts of IFN-γ transcripts. The concentration of the IFN-γ transcripts increased 2.6-fold after the avarol concentration was raised from 0.1 to 0.75 μg/ml. In the control hybridization experiments it was established that the ^{32}P-labeled vector pUC DNA did not hybridize with the isolated RNA (Fig. 4; lower row). Furthermore, incubation of the blots with the ^{32}P-labeled pBR322 vector also gave no signal on the film (data not shown).

The time-dependent appearance of IFN-γ transcripts in buffy coat cells, treated with 0.38 μg/ml avarol for 0–5 days, was analyzed quantitatively by means of dot blot hybridization experiments. The results are summarized

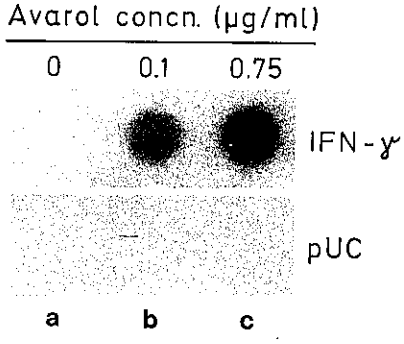

Fig. 4. Relative abundance of IFN-γ transcripts in avarol-activated lymphocytes. The cells were treated with 0, 0.1 or 0.75 μg/ml avarol for 5 days. Total RNA was isolated and 5 μg of RNA each were applied to a nitrocellulose membrane and hybridized either with ^{32}P-labeled IFN-γ DNA probe (upper panel) or with ^{32}P-labeled vector pUC DNA (lower panel). RNA from cells incubated with 0 (lane a), 0.1 (lane b) or 0.75 μg/ml of avarol (lane c).

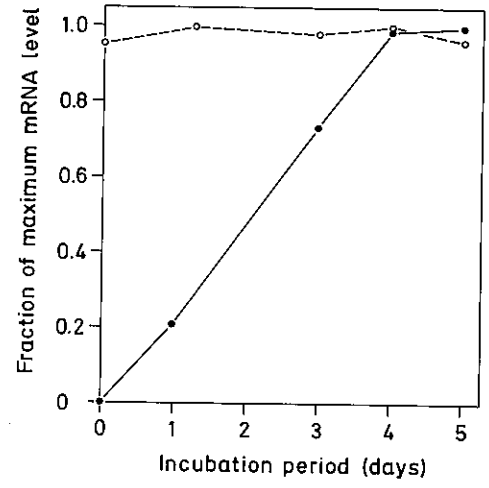

Fig. 5. Quantitative assessment of IFN-γ and actin transcripts in lymphocytes treated with 0.38 μg/ml avarol for 0–5 days. Total RNA was extracted and 3-μg aliquots were analyzed for IFN-γ and actin-specific transcripts by dot blot hybridization; the amount of RNA hybridized to the respective ^{32}P-labeled DNA probe was quantitated by scanning densitometry. The results are expressed as a proportion of maximal levels of IFN-γ (●) and actin-specific RNA (○).

in Fig. 5 and are expressed as a proportion of the maximal level for IFN-γ and actin transcripts. Again, in non-treated cells IFN-γ transcripts were absent; they appeared 24 hr after avarol addition, and reached a maximum level 4 days after activation with the compound. It should be stressed that the level of actin transcripts did not change in response to the avarol stimulus.

Change of IFN-γ mRNA Level after Avarol Treatment The relative abundance of IFN-γ mRNA was determined by Northern blot analysis of poly(A)$^+$RNA, isolated from lymphocytes that had been treated for 0–96 hr with 0.75 μg/ml avarol (Fig. 6). The results showed that, at time 0, no mRNA coding for IFN-γ was present (lane a); after an incubation period of 72 hr IFN-γ mRNAs were present (lane b) and their abundance increased further during a 96-hr incubation period.

***In vitro* Nucleocytoplasmic RNA Efflux of IFN-γ mRNA** To elucidate if the IFN-γ mRNA is transported from the nuclei to the cytoplasm by the same energy-dependent mechanism[36] which operates for at least most other mRNA species, mRNA efflux experiments were performed with nuclei *in vitro*. The lymphocytes were incubated at the optimal avarol concentration (0.75 μg/ml) for 0, 72, or 96 hr. The nuclei were prepared and transferred into a transport medium previously found to be optimal for nucleocytoplasmic mRNA efflux.[32–34] The dot blot hybridization experiments showed (Fig. 7) that no IFN-γ mRNA was released from nuclei of those cells which were not treated with avarol (lane a). On the other hand, nuclei from avarol-treated cells released significant amounts of IFN-γ mRNA (lanes b and c); the amount of IFN-γ mRNA released from nuclei of cells treated for 72 hr with avarol was approximately 35% smaller (lane b) than that obtained from nuclei of cells incubated for 96 hr with the compound (=100%; lane c). Actin mRNA was found to be released from nuclei of cells, irrespective of the existence of avarol in the incubation medium (lanes a–c). The release of both IFN-γ mRNA and actin

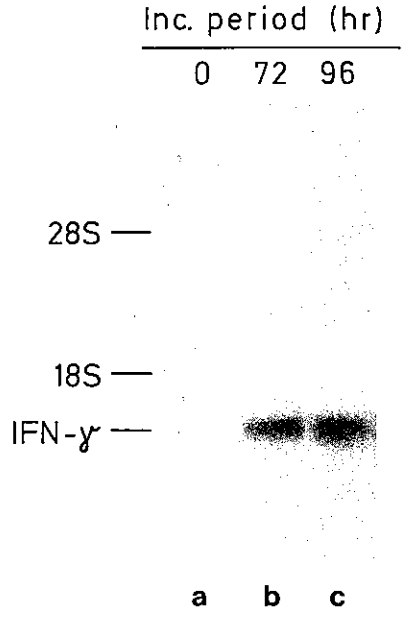

Fig. 6. Relative abundance of IFN-γ mRNA in buffy coat cells treated with 0.75 μg/ml of avarol for 0–96 hr. Total RNA was isolated and then subjected to oligo(dT)-cellulose chromatography. Poly(A)$^+$ RNA (10 μg per lane) was separated by denaturing agarose gel electrophoresis, transferred to nitrocellulose and hybridized with ^{32}P-labeled cloned IFN-γ DNA probe. Poly(A)-selected RNA from cells 0 (lane a), 72 (lane b) or 96 hr (lane c) after addition of avarol to the cultures. 18S and 28S indicate the positions of ribosomal RNA.

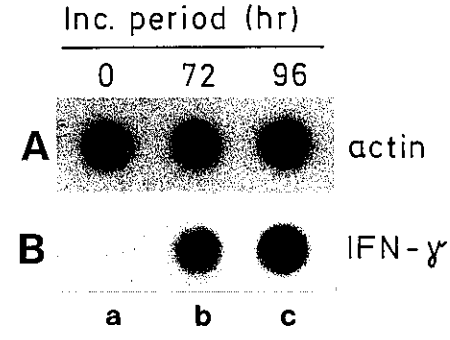

Fig. 7. Release of IFN-γ transcripts from nuclei prepared from cells incubated for 0 (lane a), 72 (b) or 96 hr (c) in the presence of 0.75 μg/ml avarol. The RNA released from the nuclei was identified by dot blot hybridization of the postnuclear supernatants with nick-translated actin (A) or IFN-γ (B) cDNA probes. The RNA efflux experiments were performed as described under "Materials and Methods."

mRNA was dependent on the presence of a nucleoside triphosphate in the efflux medium; replacement of ATP by GTP had no effect on the extent of the transport (Fig. 8; lanes a and b). In the absence of ATP no mRNA was released (data not shown). Omission of $MgCl_2$ in the transport medium resulted in an almost complete inhibition of nucleocytoplasmic mRNA transport (lanes c and d).

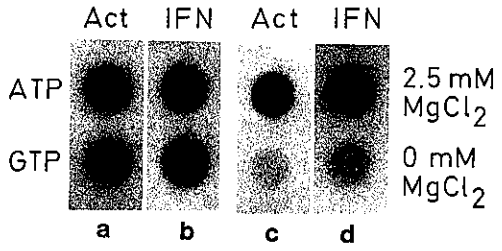

Fig. 8. Dependence of nucleocytoplasmic mRNA efflux of actin (Act) or IFN-γ (IFN) transcripts on the presence of ATP, GTP, and $MgCl_2$. Nuclei from avarol-treated cells (0.75 μg/ml for 96 hr) were incubated in the standard transport medium containing (i) 2.5m*M* ATP and 2.5m*M* $MgCl_2$ (upper panel), (ii) 2.5m*M* GTP instead of ATP (lower panel; lanes a and b) and 0m*M* $MgCl_2$ instead of 2.5m*M* $MgCl_2$ (lower panel; lanes c and d).

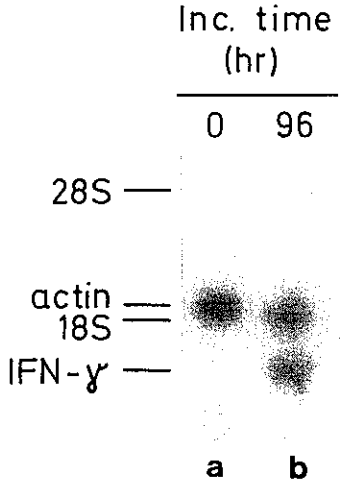

Fig. 9. RNA blot analysis of actin mRNA and of IFN-γ mRNA in RNA released from nuclei of cells, incubated for 0 hr (lane a) or 96 hr (b) in the presence of 0.75 μg/ml avarol. The RNA in the postnuclear supernatants was size-separated by gel electrophoresis, transferred to nitrocellulose and hybridized with ^{32}P-labeled actin- and IFN-γ probes. The size markers are 18S and 28S ribosomal RNAs.

The mRNA released from nuclei isolated from nontreated- and avarol-treated cells was investigated by Northern blot hybridization analysis. The results (Fig. 9) showed that IFN-γ mRNA was absent in the released RNA of nontreated cells (lane a), while it could be readily detected in the postnuclear fraction from nuclei of avarol-treated cells (lane b). The kb values of both IFN-γ mRNA and actin mRNA, released *in vitro* (Fig. 9), were identical with those found in intact cells (Fig. 3).

Discussion

In contrast to human IFN-α, which exists in at least 13 distinct non-allelic genes, only a single gene codes for IFN-γ.[23, 37] Arya and Gallo[38] showed that the IFN-γ gene is not transcribed in HIV-infected and virus-producing H9 and H4 cell lines.[38] Lymphocytes from AIDS patients were determined to produce less IFN-γ *in vitro*, after stimulation with concanavalin A or *Toxoplasma gondii*, compared to lymphocytes from healthy donors.[39] In a subsequent study it was established that this reduced capacity of IFN-γ production in lymphocytes from AIDS patients is due to an impaired antigen-induced IL-2 secretion by AIDS cells.[40] This finding suggests that in selected AIDS patients either IL-2 substitution or IFN-γ inducers might have a potentially beneficial effect against opportunistic infections.

The *in vitro* data show that avarol combines its anti-HIV effect[15] with a capacity to act as an IFN-γ inducer (this paper). Further studies are necessary to examine whether these two effects are interrelated or not. The avarol-caused IFN-γ induction was studied at both the cellular and the molecular level. Buffy coat cells were found to produce IFN-γ both dose- and time-dependently. Maximal induction of IFN-γ occurred after a 120-hr incubation period at an optimal dose of 0.75 μg/ml avarol. Also the low avarol concentration of 0.1 μg/ml caused a significant IFN-γ induction. This finding is interesting in view of previous data, which revealed that the com-

pound inhibits growth of normal lymphocytes at concentrations higher than 1–2 μg/ml.[14] However, lower concentrations were required to inhibit growth of L5178y mouse lymphoma cells (ED_{50} value: 0.3 μg/ml)[13] or to inhibit HIV replication in H9 cells (0.1 μg/ml).[15]

The enhanced production of IFN-γ was determined to be due to an induction of the IFN-γ gene in response to avarol. This molecular event is specific, at least in relation to the expression of the β-actin gene, a suitable "reference" gene for the overall transcription machinery.[41] Further studies are needed to show whether avarol causes a coordinated expression of IFN-γ gene with other genes coding for lymphokines. The presented data show that the molecular event(s) leading to an enhanced IFN-γ production occur at the level of transcription and/or post-transcription, but not at the later step of nucleocytoplasmic transport of mRNA. At present, we have no indication that avarol induces other cytokines, e.g. IFN-α, IFN-β or IL-2 (to be published).

A number of synthetic agents[42] and mitogens (e.g. concanavalin A, Staphylococcal enterotoxin, and Streptococcal preparation OK-432)[43] are capable of inducing IFNs. It is interesting that the known classes of synthetic interferon inducers, pyrimidinones and anthraquinones (see ref. 42), have striking structural similarities with avarol. These compounds, like avarol,[44] undergo an interconversion between the alcohol and the ketone form.

Presently we are working on the elucidation of the molecular basis of the described differential IFN-γ gene induction. Preliminary data show that avarol binds to the nuclear envelope and thereby modulates the activity of nuclear membrane-associated protein kinase C. Such a mechanism is conceivable in view of published findings showing that those tumor promoters which activate protein kinase C stimulate the production of IFN-γ.[45]

Acknowledgments

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