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On the Mode of Action of the Immunosuppressive Sesquiterpene Ovalicin

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When the potent immunosuppressive sesquiterpene ovalicin is added to lymphocyte cultures one first observes a preferential inhibition of uridine incorporation into rRNA. The uptake of the nucleoside, its conversion into the triphosphate or the polymerizing activity itself are not affected. A longer period of incubation with the drug results in a marked decrease in the number of ribosomes, with a concomitant reduction of the rate of leucine incorporation into all cellular proteins. After extended periods of time, the incorporation of thymidine into DNA in stimulated lymphocytes as well as in S 49.1 lymphoma cells is inhibited by 1 nM ovalicin or less, although part of the incorporation seems to be resistant to the drug even at much higher concentrations. A similar effect is observed with 3T6 mouse fibroblasts or HeLa cells. Here, however, a much longer incubation with the drug is required. This observation explains the selective effect of ovalicin on lymphocytes observed *in vivo*.

The sesquiterpene ovalicin [1–3] has been shown to be one of the most potent immunosuppressive drugs in cell culture. A drug concentration of 1–0.01 nmol/l is sufficient to inhibit significantly the antigen-induced formation of antibodies as well as the mixed lymphocyte reaction and the proliferation of murine or human lymphocytes following stimulation with various mitogens [4]. The addition of ovalicin to cell cultures several hours after the mitogen does not result in a reduction of the inhibitory effect. Therefore the very early events in the cell cycle of lymphocytes cannot be the target of the drug [4]. On the other hand, ovalicin does not reduce the incorporation of radioactive thymidine into DNA when added during the S-phase or shortly before it. Concequently DNA synthesis itself and reactions closely connected with it in time are also excluded as the targets of this inhibitor [4]. These and other observations [4] suggest that ovalicin primarily affects some reactions which occur in lymphocytes after the early phase of induction and prior to the S-phase. One of the crucial reactions in the sequence of events required for the proliferation of lymphocytes is the biosynthesis of rRNA [5] which is necessary for the formation of ribosomes. The number of active ribosmes present in resting lymphocytes is too low for cell proliferation. Mitogenic stimulation causes a 3–4-fold increase followed by attainment of a high level of protein synthesis which in turn is a prerequisite for DNA synthesis [6–9]. It therefore appeared of interest to investigate the effect of ovalicin on the synthesis of rRNA and its relationship to the synthesis of proteins and DNA.

MATERIALS AND METHODS

Cell Culture

Spleen cells from $B6D2F_1$ mice (Bomholtgard, Ry, Denmark) were cultured in Eagle's minimal medium without L-leucine (Autopow MEM, Flow Laboratories, Bonn) supplemented with 52 mg L-leucine/l and 2.5% fetal calf serum, previously heat-inactivated, in plastic flasks at a cell concentration of 2×10^6/ml. After a 12–14-h preincubation the experiments were started by addition of 1 µg concanavalin A (Pharmacia, Freiburg) per ml, 10 µM 2-mercaptoethanol and, if indicated, ovalicin (Sandoz SA, Basel, Switzerland). All added compounds were dissolved in Hank's solution.

S 49.1 lymphoma cells (obtained from U. Gehring, Heidelberg), 3T6 murine fibroblasts or HeLa cells (both obtained from E.-L. Winnacker, München) were cultured in Dulbecco's medium without non-essential amino acids in the presence of 10% fetal calf serum, 0.3 g penicillin/l, 0.1 g streptomycin/l.

Incorporation of [^{3}H]Uridine or [^{3}H]Thymidine into Acid-Insoluble Material of the Cells

At the time indicated 0.9-ml aliquots (in duplicate) of the suspended cells were transferred into small plastic tubes. 0.1 l radioactive nucleoside solution containing 1 µCi [5-^{3}H]-uridine (0.2 Ci/mmol, Schwarz-Mann, Heidelberg) or 1 µCi [*methyl*-^{3}H]thymidine (0.5 Ci/mmol, New England Nuclear, Dreieichenhain) dissolved in culture medium was added and the incubation continued as indicated. Subsequently the cells were collected on glass fibre filters (Whatman GF/C) and washed with 5% trichloroacetic acid in a similar way as described previously [10]. The radioactivity remaining on the filter was determined.

Incorporation of [^{3}H]Leucine into Acid-Insoluble Material of the Cells

At the time indicated 0.9-ml aliquots (in duplicate) of the suspended cells were transferred into small plastic tubes. The medium was replaced with leucine-free medium and 5 µCi [4,5-^{3}H]leucine (Amersham-Buchler, Braunschweig) was added (resulting specific radioactivity 0.3 Ci/mmol) and the cells incubated for 2 h. Then the cells were separated from the radioactive medium by centrifugation, washed in 2 ml cold phosphate-buffered saline containing 0.1% NaN_3 and subsequently lysed in 0.2 ml 1 M NaOH at 56 °C for 10 min. The protein was precipitated with 2 ml 10% trichloroacetic acid containing 0.13 mg L-leucine/ml and collected after 1 h

Dedicated to Professor Helmut Holzer on the occasion of his 60th birthday.

on to glass-fibre filters (Whatman GF/C). After further washing with trichloroacetic acid and methanol the radioactivity remaining on the filter was determined.

Pulse Labelling of RNA in Stimulated Lymphocytes

Two cultures with 3×10^7 lymphocytes were stimulated as described, one of the cultures in the presence of 0.2 μM ovalicin; 12 h later 3.45 μM [^{3}H]uridine (2 μCi/ml) was added to the culture without the drug, whereas the same concentration of [2-^{14}C] uridine (Schwarz-Mann, Heidelberg, 0.2 μCi/ml) was added to the culture containing the drug. Both cultures were incubated for 1 h. The concentration of uridine was subsequently increased to 1 mM with unlabelled nucleoside for 30 min in the pulse-chase experiment. Simultaneously 5 μg of actinomycin D/ml was added. Then both cultures were combined and protein denatured with phenol in the presence of dodecylsulfate [11]. RNA, precipitated with ethanol, was dissolved in buffer [12] and separated electrophoretically in polyacrylamide gels containing 0.5% agarose [12]. To determine of the radioactivity the gels were cut into 1-mm slices which were incubated overnight by shaking in Soluene 350 (Packard, Frankfurt) at room temperature and, subsequently, for 1–2 h at 50 °C. After addition of the scintillation cocktail the radioactivity of ^{14}C and ^{3}H was measured separately; the measured values were corrected for spillover.

Cell-Free System for the Incorporation of [^{3}H]UMP into RNA

Nuclei were prepared by treatment of 10^8 lymphocytes, which had been stimulated for 36 h (control without concanavalin A) with or without 0.2 μM ovalicin, with 0.25 M sucrose containing 5 mM $CaCl_2$, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Serva, Heidelberg) pH 8.0 and 0.5% Brij 58 (Serva, Heidelberg) as described [13] and used directly for the assay. The incorporation of [^{3}H]-UMP from [^{3}H]UTP into RNA of the isolated nuclei was measured essentially as described [14] with 2×10^6 nuclei per assay in the presence or absence of 20 μM ovalicin.

Electrophoretic Analysis of ^{14}C-Labelled Proteins

The cells which had been incubated with 1 μCi/ml of [^{14}C]leucine (0.11 Ci/mmol, Schwarz-Mann, Heidelberg) were separated from the medium by centrifugation, washed with 10 ml 0.85% NaCl and frozen. After thawing the cells were dissolved in 40 μl 50 mM Tris/HCl pH 6.8 containing 2.5% sodium dodecylsulfate, 8% glycerol and 5% 2-mercaptoethanol; 20-μl aliquots were applied to slab gels [15] with a linear 5–15% acrylamide gradient. Electrophoresis was carried out for 16 h at 60 V. Autoradiography was performed with DuPont Cronex 2 X-ray screen film NIF 200 for 7 days.

Determination of Ribosomes in Stimulated Lymphocytes

Freshly isolated lymphocytes (1.1×10^8) were stimulated with concanavalin A. One culture contained in addition 0.2 μM ovalicin. After 23 h 10 μM 2-mercaptoethanol was added and the incubation continued for 17 h. Then 0.9-ml aliquots (in duplicate) were removed to determine the rate of incorporation of [^{3}H]leucine (5 μCi/ml; 13 Ci/mol) into acid-insoluble material with a 4-h pulse in a similar way to that described previously [16]. The remaining cells were used to isolate the ribosomes as described [17]. The amount of ribosomes was characterized by the content of RNA as determined by the absorbance at 260 nm [18].

RESULTS

Incorporation of Uridine

Previously we have shown that, in activated lymphocytes, the incorporation of radioactive uridine into acid-insoluble material is partly inhibited by ovalicin [4]. The effect of the drug was studied in a cell-free system to prove that this inhibition reflects a decrease in RNA synthesis and not a reduction of the uptake of uridine, an inhibition of its conversion into ribonucleoside triphosphate or a change of the pool size of the precursor. The incorporation of [^{3}H]UTP into nuclei from lymphocytes activated by concanavalin A either in the presence or absence of ovalicin was determined (Table 1). As can be seen, nuclei obtained from cells treated with ovalicin show a markedly reduced incorporation of nucleotide, indicating that the drug inhibits the incorporating system or its formation and not the uptake by or metabolism of the precursor in intact cells. When the drug was added to nuclei obtained from cells stimulated in the absence of ovalicine no reduction of the incorporation was found (Table 1). This indicates that ovalicin does not directly interfere with the catalytic process of the polymerizing system. This conclusion, however, rests on the assumption that the active agent is the drug itself and not a metabolised form of it.

The observation that in cell culture the extent of the inhibition is independent of the length of the uridine pulse (between 5–60 min, data not shown) also supports the notion that uptake and metabolism of the precursor are insensitive to the action of ovalicin. In agreement with this conclusion the apparent Michaelis constant for uridine during the incorporation into RNA (K_m = 2.95 μM) is not influenced by ovalicin. It therefore seems likely that the reduced labelling of RNA in the presence of ovalicin is either due to an inhibition of its biosynthesis or a stimulation of its degradation.

For a closer analysis of the effect of the drug on the metabolism of rRNA, the following experiment was carried out. Concanavalin A was added to a culture of resting murine spleen lymphocytes together with ovalicin, which was omitted in the control; 12 h later [^{14}C]uridine was added to the culture containing the drug, whereas [^{3}H]uridine was given to the control. The labelling period of 1 h was followed by a 0.5 h chase with a 300-fold excess of unlabelled uridine. Actinomycin D was simultaneously added to prevent the incorporation of labelled nucleotides released during the degradation of radioactive RNA [19]. Both cultures were subsequently combined, RNA was extracted and analysed by electrophoresis in a polyacrylamide gel. Four radioactive peaks are observed corresponding to 28-S, 18-S, 5-S and 4-S RNAs as calculated from the mobility of marker RNAs (Fig. 1 A). Almost no radioactive RNA larger than 28 S was observed in agreement with the fact that large hnRNA is rather unstable with a half life of only 8 min [20]. The determination of the ratio [^{3}H]uridine/[^{14}C]uridine in the gel clearly shows that ovalicin inhibits the incorporation of the labelled precursor into the three species of rRNA to a much grater extent than into 4-S RNA (Fig. 1 B). The inhibition is not complete and amounts to 35–44% (Table 2). This is not due to an insufficient time of incubation with the drug (in these experiments 12 h) since a constant level of inhibition is reached in a much shorter time as will be shown below

Table 1. *Incorporation of [^{3}H]UMP into nuclei from lymphocytes stimulated in the presence or absence of ovalicin without or with further addition of the drug*

Spleen lymphocytes were cultured for 36 h in the absence or presence of 0.2 μM ovalicin. Subsequently nuclei were prepared and the incorporation of [^{3}H]UMP from [^{3}H]UTP at 25 °C for 60 min into acid-insoluble material was determined. The value of a control with zero-time incubation (1268 counts × min^{-1}) has been substracted. The data are mean values of determinations performed in duplicate which differed by less than 6%

Preincubation of cells		Incubation: 20 μM ovalicin added	[^{3}H]UMP incorporation per 2×10^6 nuclei
mitogen	ovalicin		
			counts × min^{-1}
−	−	−	441
+	−	−	6494
+	−	+	7535
+	+	−	3641

Table 2. *Inhibition by ovalicin of the labelling of rRNA with radioactive uridine in stimulated lymphocytes*

The inhibition was calculated from the data shown in Fig. 1 as follows. If the incorporation of [^{3}H]UMP in the presence or absence of ovalicin is designated as a and a', respectively, and that of [^{14}C]UMP as b and b', respectively, the inhibition I (in %) by the drug of the incorporation of the uridine nucleotide into a particular fraction r of RNA can be calculated from the measured ratio $(a'/b)_r$ (see Fig. 1B) by the equation $I = 100\ [1 - F/(a'/b)_r]$, where F is the ratio $a'/b' = a/b$, given by the specific radioactivity of the labelled nucleotides incorporated and the mixing ratio of the two incubation samples (Fig. 1), which is independent of the particular RNA studied. The ratio a/b was determined prior to the electrophoretic fractionation as 5.75 ± 0.38 from $a/a' = 0.664 \pm 0.020$ and $a'/b = 8.67 \pm 0.30$ (mean values of two determinations) according to $a/b = (a/a')(a'/b)$

rRNA species	[^{3}H]UMP/[^{14}C]UMP incorporated	Inhibition
		%
28 S	8.8 ± 0.3	35 ± 6
18 S	10.3 ± 0.3	44 ± 6
5 S	9.0 ± 0.3	36 ± 6

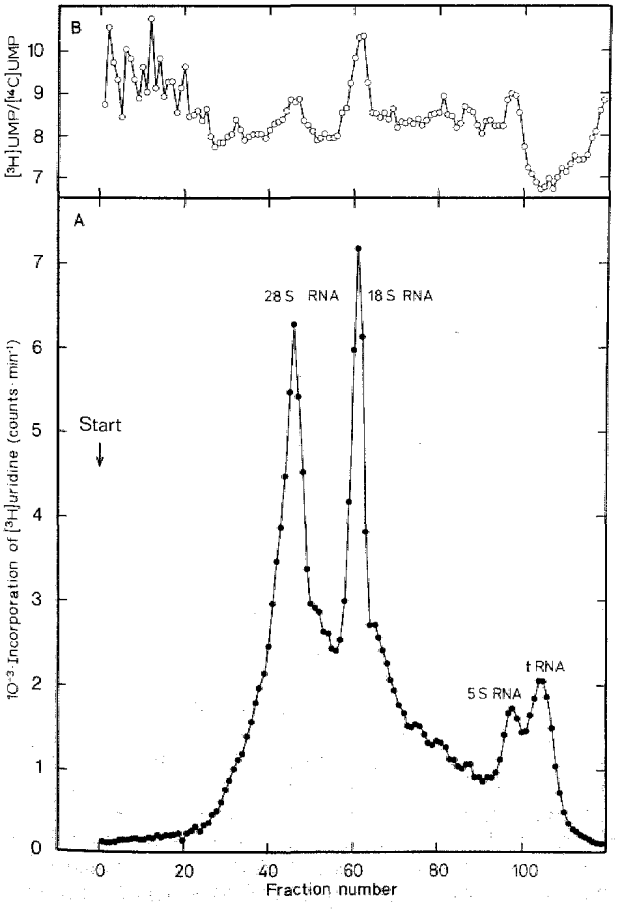

Fig. 1. *Influence of ovalicin on the incorporation of uridine into rRNA in stimulated lymphocytes.* (A) As measured in a pulse-chase experiment (electrophoretic analysis in gels prepared with 2.2% acrylamide; acrylamide:*N*,*N*′-methylene-bisacrylamide = 48:1). (B) Ratio of [^{3}H]UMP/[^{14}C]UMP incorporated into a particular fraction of RNA

(Fig. 3). The incomplete inhibition could be due to the use of a spleen cell culture which contains, among other cells, several classes and subclasses of lymphocytes with possibly different sensitivities to the drug. This question will be investigated in future work using cell lines. The labelling of the individual rRNAs appears to be inhibited by ovalicin to a somewhat different extent (Table 2). Similar observations have been made in other experiments without ovalicin and explained in different ways [21, 22].

In order to study the effect of ovalicin on the labelling of the unstable hnRNA the experiment was repeated; however, this time the chase with unlabelled uridine was omitted. In subsequent electrophoretic analysis polyacrylamide gels with a higher degree of cross-linking were used for better separation of large hnRNA from rRNA. Again, the labelling of rRNA is clearly inhibited by the drug. In contrast the labelling of the high-molecular-weight RNA remaining at the start is much less reduced (Fig. 2). Assuming that the precursor for the synthesis of the various species of RNA is derived from the same pool, these results suggest a specific effect of ovalicin on the metabolism of rRNA. In agreement with the observation that the metabolism of rRNA is insignificant in resting lymphocytes [20], the inhibition of the labelling of RNA in these cells is very small (7.5 ± 6.2%) even after a 19-h incubation with ovalicin (data not shown).

The DNA-dependent RNA polymerase I responsible for the biosynthesis of the rRNA precursor can be ruled out as the target of ovalicin since no effect of the drug on RNA polymerase I purified from murine MOPC cells is detectable *in vitro* (W. A. Zimmermann, unpublished observations).

In the experiments described above, the inhibitory effect is observed after exposing the cells to the drug for 12 h. If the metabolism of rRNA is rather closely connected in time with the molecular target of the drug, the inhibition should be observable after a shorter time of exposure. To answer this question spleen cells were stimulated with mitogen. Ovalicin was then added at different times; 12 h after addition of the mitogen the labelling of RNA was measured with a 1-h pulse of [^{3}H]uridine. In agreement with the previous experiments an inhibition of 35% was observed after a long incubation with the drug (Fig. 3). But even when ovalicin was added only 1 h before the pulse a distinct inhibition (50% of the

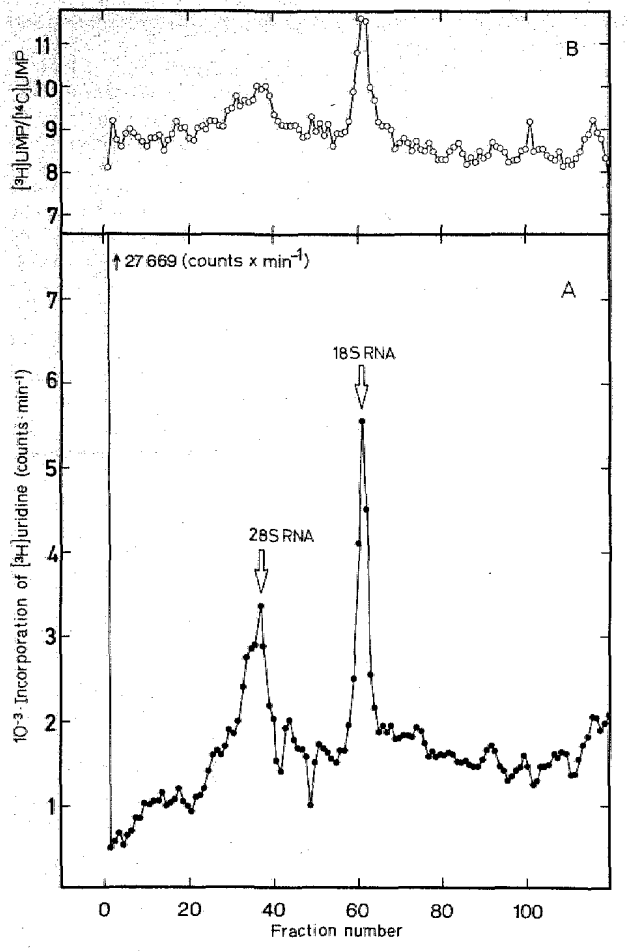

Fig. 2. *Influence of ovalicin on the incorporation of uridine into RNA in stimulated lymphocytes.* (A) As measured without a chase with unlabelled uridine (electrophoretic analysis in gels prepared with 2.2% acrylamide; acrylamide:*N,N'*-methylene-bisacrylamide = 20:1). (B) Ratio of [^{3}H]-UMP/[^{14}C]UMP incorporated into a particular fraction of RNA

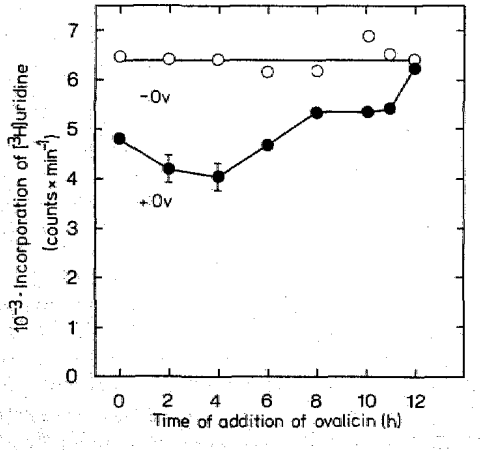

Fig. 3. *Kinetics of the inhibitory action of ovalicin on the incorporation of [^{3}H]uridine into RNA of stimulated lymphocytes.* Ovalicin (Ov, final concentration 0.2 µM) in balanced salt solution was added at the times indicated on the abscissa. Simultaneously the control cultures received an equal amount of balanced salt solution. Incorporation of [^{3}H]uridine during a 2-h pulse was determined 12 h after addition of the mitogen: (○) without drug; (●) with drug

Table 3. *Effect of ovalicin on the content of ribosomes and on the rate of incorporation of [^{3}H]leucine into protein in stimulated lymphocytes*
The incubation of lymphocytes, pulse-labelling with [^{3}H]leucine, isolation of ribosomes and determination of rRNA content was performed as described in Materials and Methods. The data are mean values of determinations performed in duplicate

Ovalicin	Ribosomes	Incorporation of [^{3}H]leucine during 4 h into 2.07×10^7 cells
µM	µg rRNA/2×10^8 cells	counts × min^{-1}
0.2	168 ± 8	11968 ± 553
0	320 ± 15	22001 ± 189

maximum) was found. This effect of the drug is the earliest one detectable so far. Consequently we have to conclude that the metabolism of rRNA must be rather closely connected with the molecular target of ovalicin.

Incorporation of Leucine

Any reduction of the synthesis of rRNA should lead to a decrease in the formation of new ribosomes. Indeed, when the content of ribosomes in lymphocytes is determined, we find that cells stimulated in the presence of ovalicin contain half as many ribosomes as the control cells without the drug (Table 3). A reduction in the number of ribosomes should lead to a decrease in the rate of protein synthesis, which can be measured by the incorporation of radioactive leucine into proteins since exogenous leucine readily equilibrates with intracellular leucine. Uptake and the size of the pool of free amino acid do not change significantly after stimulation of the lymphocytes [7,23,24]. When the incorporation of [^{3}H]-leucine was measured in parallel with the content of ribosomes, it was found that the incorporation in the presence of ovalicin was only 50% of that of the untreated control (Table 3).

If the reduction of protein synthesis by ovalicin is caused by the decrease in the number of ribosomes, the synthesis of all proteins should be uniformly affected. To test this notion murine spleen lymphocytes were stimulated with mitogen in the presence or absence of ovalicin. At various times after stimulation, proteins were labelled by a 4-h pulse with [^{14}C]-leucine. Subsequently the cells were dissolved in buffer containing dodecylsulfate and the proteins fractionated by gel electrophoresis. The incorporation of radioactive leucine into the numerous zones of protein was monitored by autoradiography (Fig. 4). The incorporation of radioactivity increased with the time of exposure to the mitogen. This is particularly evident for the most rapidly migrating zones which include the proteins required for DNA replication such as histones [25]. After a longer period of incubation less radioactivity was incorporated in the presence of the drug. Nevertheless the pattern and the ratio of the intensities of the radioactive zones within a slot was the same in the presence and absence of the drug. A similar result was found when the sensitive double-labelling method was used (data not shown). These results suggest that ovalicin inhibits uniformly the biosynthesis of the various classes of proteins.

Finally it is of interest to determine the kinetics of inhibition of leucine incorporation with that of uridine incorporation. Murine spleen cells were stimulated with mitogen in the presence of ovalicin. Controls without the drug and (or)

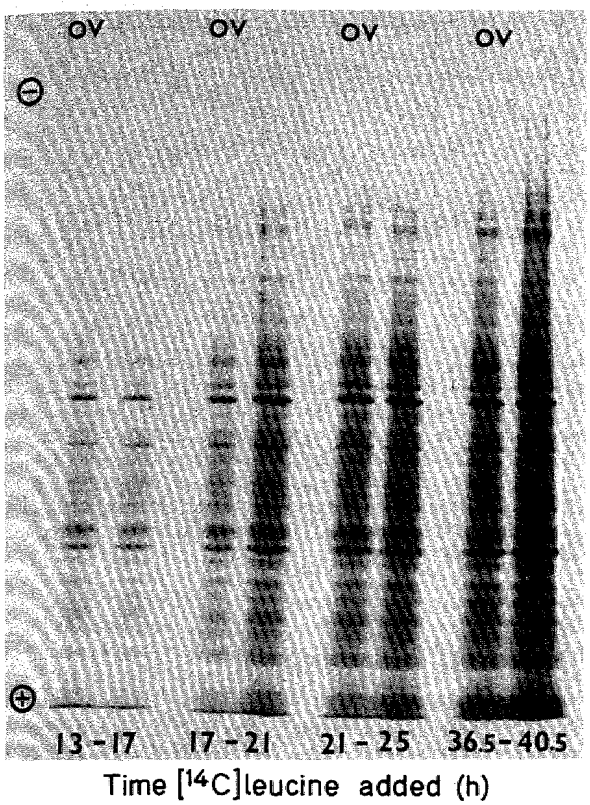

Fig. 4. *Effect of ovalicin on the incorporation of [^{14}C]leucine into different fractions of protein in stimulated lymphocytes.* The radioactive amino acid was given to the cells at different times (as indicated) after addition of 0.2 μM ovalicin (ov) and concanavalin A for 4 h. The incorporation was visualized by autoradiography of the dried gel after electrophoresis. To increase the specific radioactivity of the labelled amino acid the cells were washed with leucine-free medium prior to the addition of 1 μCi [^{14}C]leucine (resulting specific radioactivity 0.11 Ci/mmol)

without mitogen were run in parallel. At different times after the start, the incorporation of [^{3}H]leucine into acid-insoluble material was measured during a 2-h pulse (Fig. 5). Ovalicin reduces the incorporation by 15% after an 11-h incubation. A really significant inhibition was only observed much later. When comparing these results with the kinetics of the inhibition of uridine incorporation (Fig. 3) we have to conclude that the inhibition of leucine incorporation takes distinctly longer.

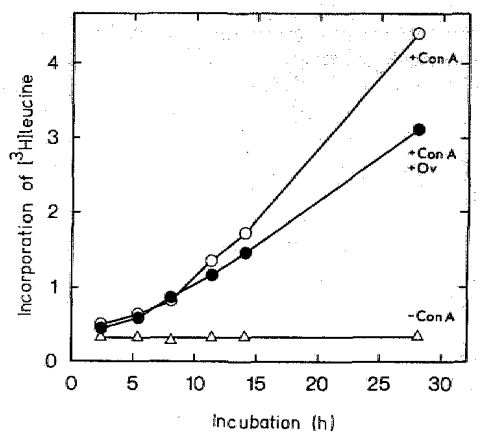

Fig. 5. *Kinetics of the inhibitory action of ovalicin on the incorporation of [^{3}H]leucine into protein of stimulated lymphocytes.* The experiment was started by addition of concanavalin A (Con A), 2-mercaptoethanol and 0.2 μM ovalicin (Ov) to a culture with 4×10^7 cells (●); controls without ovalicin (○) or without mitogen and drug (△). At 2 h prior to the time indicated on the abscissa, 0.9-ml aliquots (in duplicate) were transferred to small plastic tubes to determine the incorporation of [^{3}H]leucine into acid-insoluble material

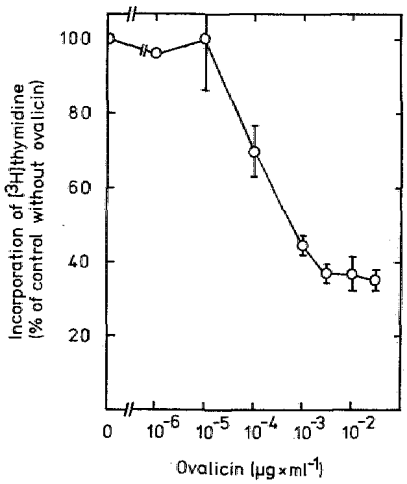

Fig. 6. *Effect of different concentrations of ovalicin on the incorporation of [^{3}H]thymidine into DNA of stimulated lymphocytes.* At 30 h after addition of the mitogen and ovalicin, 0.9-ml aliquots (in duplicate) were transferred to small plastic tubes to determine the incorporation of [^{3}H]-thymidine during a 1-h pulse into DNA (100% = 20896 counts × min^{-1})

Incorporation of Thymidine

It has been reported that the incorporation of radioactive thymidine into DNA is also inhibited by ovalicin, although a considerable time of incubation is required to achieve a substantial effect [4]. This inhibition is not caused by a significant change in the uptake, the metabolism or the size of the pool of the precursor since it is also observed in a cell-free system of nuclei where dTTP serves as precursor [4]. This notion is supported by the observation that the extent of inhibition caused by the drug is independent of the concentration of thymidine used (data not shown). Therefore we conclude that the inhibition of thymidine incorporation by ovalicin reflects an effect on the synthesis of DNA.

The dose-response curve of this effect (Fig. 6) shows two interesting features: (a) a maximum of inhibition is reached with less than 1 ng ovalicin/ml; (b) 35% of the incorporation is resistant to the drug even when its concentration is increased 50-fold. This suggests that the resistant incorporation may be a consequence of using an unfractionated spleen cell culture containing several classes of lymphocytes with possibly different sensitivities to the drug. It was therefore of interest to investigate the effect of ovalicin on monoclonal lymphoid cells such as S 49.1 lymphoma cells [26] (Fig. 7A). In this experiment S 49.1 cells were incubated for various periods of time (starting from almost one cell cycle up to three cell cycles) with different concentrations of ovalicin. Subsequently the incorporation of [^{3}H]thymidine into DNA was determined during a 1-h pulse. It is evident that S 49.1 cells were inhibited at the same low concentration of ovalicin as

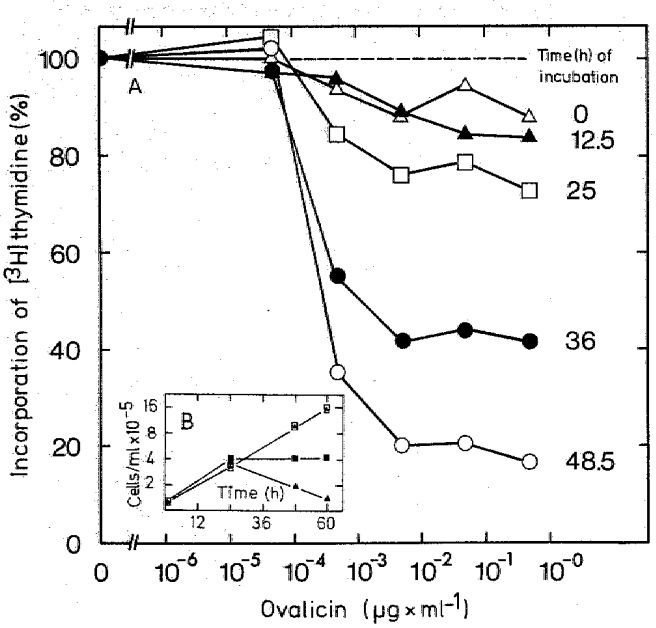

Fig. 7. *Effect of different concentrations of ovalicin and of the time of incubation on the incorporation of [^{3}H]thymidine into DNA and on the cell number of a S 49.1 lymphoma cell culture.* (A) To exponentially growing cells ($0.15-0.3\times10^6$ cells/ml) ovalicin (as indicated on the abscissa) was added. After the time indicated in the figure, 0.9-ml aliquots (in duplicate) were transferred to small plastic tubes to determine the incorporation of [^{3}H]thymidine into DNA during a 1-h pulse: 100% = 11084 (△), 23213 (▲), 39115 (□), 56431 (●) or 70820 counts × min^{-1} (○). (B) Exponentially growing cells were diluted with fresh medium containing 0.2 µM ovalicin to 0.15×10^6 cells/ml. At the time indicated on the abscissa the number of cells and the number of vital cells (as measured by the exclusion of trypan blue) was determined microscopically. Total cell number: (□) without ovalicin; (■) with ovalicin; vital cells: (△) without ovalicin; (▲) with ovalicin

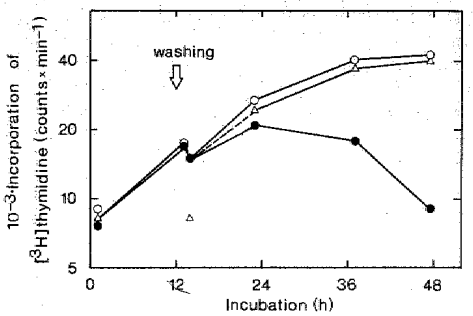

Fig. 8. *Reversibility of the inhibitory action of ovalicin on S 49.1 lymphoma cells.* 20 nM ovalicin was added to exponentially growing cells ($0.15-0.3\times10^6$ cells/ml); (○) controls without drug. 12 h later the cells were removed from the medium by centrifugation, washed and resuspended in fresh medium without the drug (△) (resulting concentration of ovalicin in the medium 20 pM) or in medium with 20 nM drug (●). 1 h prior to the time indicated, 0.9-ml aliquots (in duplicate) were transferred to small plastic tubes to determine the incorporation of [^{3}H]thymidine into DNA during a 1-h pulse

spleen lymphocytes. Again, part of the incorporation of thymidine into DNA is resistant to the drug irrespective of its concentration. However, the extent of resistance is strongly dependent on the time of incubation of the cells with ovalicin. A rather short incubation (less than one cell cycle) leads to low inhibition. Only after a much longer incubation was the incorporation substantially reduced. If one determines the number of cells in such an experiment, it becomes obvious that ovalicin has almost no effect for the first 24 h (Fig. 7B). Upon further incubation the total number of cells remains constant but cells start to die and become permeable for trypan blue. Obviously the inhibition of proliferation by ovalicin is a late effect in agreement with the findings in stimulated spleen lymphocytes [4].

Indeed all short-term effects of ovalicin seem to be completely reversible up to 12 h of incubation as is suggested from the following experiment. Ovalicin was added to exponentially growing S 49.1 cells and incubation was continued for 12 h. Then the concentration of the drug was lowered to 0.02 nM by washing the cells with fresh medium where upon the incubation was continued. The incorporation of thymidine into DNA was measured by 1-h pulses at various times (Fig. 8). The rate of incorporation remained the same as in the control without the drug. When the drug was not removed the incorporation was strongly inhibited upon further incubation.

Effects on Non-lymphoid Cells

From the comparison of the influence of ovalicin on the number of mitoses in different cells [2], it is known that non-lymphoid cells appear to be much less susceptible to the action of this drug. Therefore we became interested in investigating its action on the incorporation of thymidine into cultures of 3T6 mouse fibroblasts and human HeLa cells. It is evident that the inhibition was but small but it increased with the time of incubation (Fig. 9A) and, interestingly, became evident at the same low concentration of ovalicin as in lymphocytes (Fig. 9B). The unusually low concentration of the drug required for inhibition suggests a similar mode of action in all eukaryotic cells tested. However, in all rapidly dividing cells the inhibition is detected only after an incubation of the cells with ovalicin for more than one cell cycle (Fig. 7 and 9).

DISCUSSION

The first detectable effect of ovalicin on stimulated lymphocytes is the specific inhibition of the incorporation of radioactive uridine into rRNA (Fig. 3). Upon a longer period of incubation the number of ribosomes per cell and the rate of incorporation of radioactive leucine into proteins and of labelled thymidine into DNA is also reduced. All these effects including those occurring after a long exposure of the cells to the drug are observed with very low concentrations of the inhibitor. It is important to stress this fact because at higher concentrations hydrophobic drugs such as ovalicin can bind to many different sites in the cell by rather unspecific hydrophobic interactions with the consequence of inhibiting several reactions separately and independently as has been proven for the rifamycins [27, 28] or novobiocin and nalidixic acid [29]. At such low concentrations of a drug (100–0.1 nM) as were used in our experiments unspecific binding has not been reported so far for other drugs. Therefore it appears reasonable to assume a single target for ovalicin. Its blockade could then lead via a chain of metabolic events to the inhibition of many other reactions. This hypothesis is supported by the kinetics of the various inhibitory effects. Except for the

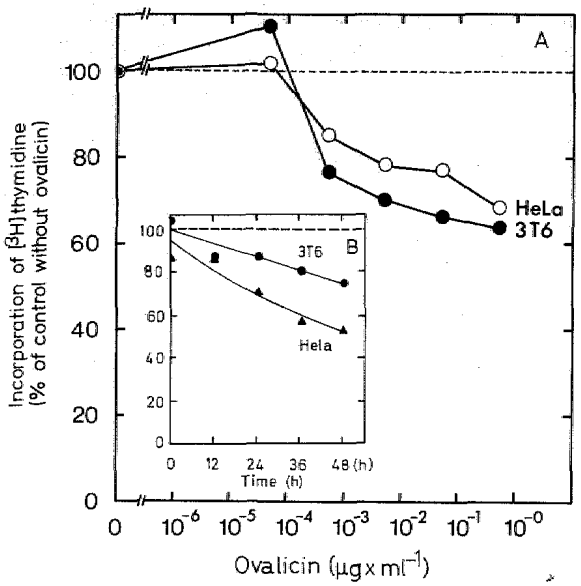

Fig. 9. *Effect of different concentrations of ovalicin on the incorporation of [^{3}H]thymidine into DNA of HeLa cells or 3T6 murine fibroblasts.* (A) Ovalicin (as indicated) was added at 6 h to HeLa cells (○) or at 9 h to 3T6 cells (●) after suspending the cells in 2 ml fresh medium. 48 h after addition of the drug, 2 μCi [^{3}H]thymidine (specific radioactivity 0.5 Ci/mmol) was added and the incubation continued for 1 h; 100% incorporation = 1332 counts × min^{-1} for HeLa cells or 12861 counts × min^{-1} for 3T6 cells. (B) Ovalicin was added as in (A). 1 h before the time indicated on the abscissa 2 μCi [^{3}H]thymidine was added to (▲) HeLa cells (specific radioactivity 6 Ci/mmol) or (●) 3T6 cells (specific activity 0.1 Ci/mmol) and the incubation continued for 1 h. 100% values in counts × min^{-1} were for HeLa cells: 4552 (1 h), 7624 (13 h), 11208 (25 h), 23404 (37 h) and 33910 (49 h); for 3T6 cells: 632 (1 h), 1390 (13 h), 2820 (25 h), 5010 (37 h) and 9930 (49 h)

inhibition of the incorporation of uridine into RNA all other effects are observed only after several hours of incubation. It is not yet known how ovalicin on the molecular level, specifically reduces the formation of 28-S, 18-S and 5-S rRNAs, but this will be the subject of further studies. During the stimulation of the lymphocytes the rate of synthesis of rRNA increases more than that of hnRNA [5, 20]. As a consequence the total number of ribosomes in lymphocytes increases 3–4-fold during the stimulation [6, 7] with a concomitant increase in the capacity to synthesize proteins. This, however, is not the initial mechanism stimulated lymphocytes use to increase their rate of protein synthesis. Rather, the primary step is the activation of inactive ribosomes preexisting in the cell [30, 31]. In agreement with these findings the formation of blast cells from small lymphocytes, a process which requires protein synthesis, is only weakly reduced by ovalicin [4]. For the subsequent process of proliferation the number of ribosomes in the cell may be rate-limiting [32]. This is not unexpected because DNA synthesis in eukaryotic cells requires a very active and simultaneous synthesis of proteins, particularly of histones which are necessary for the formation of chromatin [9]. Therefore, a reduction of the number of newly formed ribosomes by ovalicin should also diminish the capacity to synthesize DNA as has been reported [4]. The very same reasoning explains why the inhibition of the incorporation of thymidine into DNA by ovalicin is never complete. The ovalicin-resistant DNA synthesis may be simply the consequence of protein synthesis on preexisting ribosomes in the lymphocyte.

Another explanation for the partial resistance could be based on the existence of several populations of cells in the culture which differ in the content of ribosomes, implying a critical number of ribosomes for the initiation of DNA synthesis. This hypothesis also explains the apparent insensitivity of non-lymphoid cells to ovalicin, at least when these cells are incubated with the drug for only one cell cycle. Only resting lymphocytes are particularly deficient in ribosomes and have to increase the number upon stimulation before the S-phase can be entered for the first time [6, 7]. In contrast, rapidly proliferating cells such as lymphoma cells, HeLa cells or fibroblasts apparently contain a sufficient number of ribosomes to initiate chromatin synthesis. Consequently these cells can proliferate even in the presence of inhibitors of tRNA synthesis such as small doses of actinomycin D for more than one cell cycle before the number of ribosomes becomes rate-limiting [32–34]. Consequently such cells resist the action of ovalicin for longer times than stimulated lymphocytes (Fig. 7 and 9).

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