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ANTIVIRAL AND CELL MULTIPLICATION INHIBITORY ACTIVITIES OF MOUSE INTERFERON PREPARATIONS TESTED ON AN INTERFERON SENSITIVE MURINE SARCOMA CELL LINE

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The cell multiplication inhibitory effect of SDS-treated mouse interferon separated into antiviral (AV) and cell multiplication inhibitory (CMI) fractions was compared to that of untreated similar interferon on a line of murine osteosarcoma cells. The untreated interferon preparation and the CMI fractions dose-dependently inhibited the multiplication of the cells as measured by cell count and incorporation of ^{3}H-thymidine into the cultures. The AV fractions, containing comparable antiviral activites as the untreated interferon preparations, had only a minor effect on cell multiplication. The biochemical properties of the fractions studied remain unknown.

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Besides its well-documented antiviral activity, interferon exerts several biological effects at the cellular level (17). Among these »non-antiviral« activites the antitumor effect (13) and the cell-multiplication inhibitory effect (7, 12, 16) have been most extensively studied. Interferon preparations inhibit the multiplication of both normal and tumor cells *in vitro* (2, 7, 12, 15, 16), and the development of tumors *in vivo* (13) in several experimental models. *Glasgow* and co-workers (3, 11) reported that interferon inhibited murine osteosarcomas *in vitro* and *in vivo*.

In most studies the antiviral and the cell-multiplication inhibitory activities of interferon correlated strictly. However, separation of these activities has also been reported: first by affinity chromatography on an albumincoupled agarose column (6) and subsequently by boiling interferon preparations in the presence of sodium-dodecyl-sulfate (SDS) followed by gel filtration on a Sephadex G-25 column (4).

In the present study the effects of fractions obtained by the SDS method on murine osteosarcoma cells *in vitro* have been studied.

MATERIALS AND METHODS

Cells:

The continuous line of mouse L-929 fibroblast cell, originally obtained from the American Type Culture Collection, Rockwille, Md, was grown in Eagle's minimal essential medium (MEM) supplemented with 5–10 per cent fetal bovine serum, 300 µg glutamine, 100 µg penicillin and 50 µg streptomycin per ml.

A mouse osteosarcoma cell line established from a ^{239}Pu irradiation produced primary tumor in C57B1/6 mice (11). The osteosarcoma cells were grown in the same medium as the L cells. In the present experiments the cells were in their 50–70th passage in cell culture. The cells remained tumorigenic when injected into C57B1/6 mice.

Interferon:

Murine type I interferon preparation, produced by Dr *K. Paucker,* Medical College of Pennsylvania, Philadelphia, Pa, by exposing L- cells to Newcastle disease virus (NDV), was received from the antiviral program of the National Institute of Allergy and Infectious Diseases, Bethesda, Md. The specific activity of this preparation was about 1×10^7 units per mg protein. Another preparation, produced in L- cells by NDV inoculation, was given to us by Dr *G. Bodo,* E. Boehringer Institut für Arzneimittelforschung, Vienna. This preparation contained 1.3×10^6 units of interferon per mg protein. Antiviral activity was measured against Vesicular stomatitis virus (VSV) in L cells by a plaque reduction method (14) or by an infectivity inhibition microtest (5). All titres were compared to international reference standards.

Interferon fractions containing antiviral (AV) and cell multiplication inhibitory (CMI) activities, prepared by SDS treatment, were obtained from *H. Dahl,* Wilhelmsen Institute, University of Oslo. Briefly, interferon preparation was treated with 3.3×10^{-3} M SDS in boiling water bath for 1 minute. After 1 hour at room temperature the preparation was gel filtrated on Sephadex G-25 column, and the antiviral and cell multiplication inhibitory activites were tested in each fraction (4, 7). The active fractions were pooled for further studies.

Determination of Cell Proliferation

Osteosarcoma cells were seeded in 24 well plates (Linbro), 5×10^3 cells in each well. Interferon or interferon fractions obtained by SDS treatment were added simultaneously and incubated at 37 °C. After various times of incubation the cells were trypsinized, and the number of viable cells was determined after trypan blue staining in a haemocytometer. Each value given in the results is the mean of three or four determinations. Other cultures were fixed with 20 per cent formalin at the end of incubation, washed and stained with methylene blue.

Incorporation of ^3H-thymidine (Tdr) into Cell-cultures

Osteosarcoma cells were seeded in 96 well plates (Linbro), about 5×10^3 cells into each well. Interferon or interferon fractions were added simultaneously. The cells were incubated at 37 °C for 24 or 72 hours. At each time period 1 μCi ^3H-Tdr (40–60 μCi/mM, New England Nuclear, Boston, Ma) was added to each well and incubated for 4 hours. The supernatant was then discarded, the cells were washed with phosphate buffered saline (PBS), trypsinized and harvested onto filter pads with the aid of an automated cell harvester (Scatronic, Flow Laboratories Inc., Inglewood, Ca). Filterpads were

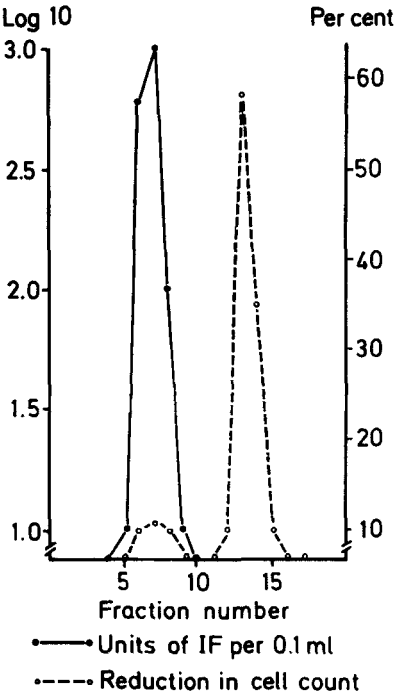

Fig. 1. Antiviral (AV) and cell multiplication inhibitory (CMI) activities of fractions obtained from gel filtration on Sephadex G 25 of mouse interferon treated with sodium dodecyl sulfate at 100 °C for 1 minute. L-929 cells were grown in the presence of fractions for 3 days. Each fraction was tested at dilution 1:25.
●———● Units of interferon per 0.1 ml.
O------O Per cent reduction in cell count.

TABLE 1. *Effect of Mouse Interferon and the Cell Multiplication Inhibitory and Antiviral Fractions on the Multiplication of Osteosarcoma Cells*

Preparation, Antiviral activity		Cell count after 3 days of incubation $\times 10^3 \pm$ SD
Control cells		33.3 ± 6.81
Interferon,	400 u/ml	11.6 ± 2.08
Interferon,	10 u/ml	15.6 ± 4.16
AV fraction, (1:10 dilution)	1000 u/ml	23.6 ± 2.10
AV fraction, (1:25 dil.)	400 u/ml	28.3 ± 1.53
AV fraction, (1:100 dil.)	10 u/ml	29.3 ± 1.58
CMI fraction, (1:25 dil.)	1 u/ml	13.0 ± 2.08
CMI fraction, (1:100 dil.)	<1 u/ml	22.0 ± 0.

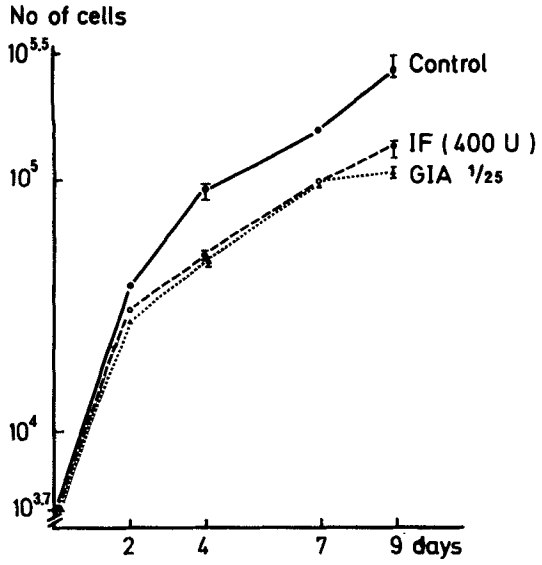

Fig. 2. Growth of osteosarcoma cells in the presence of interferon and the CMI fraction. Vertical bars indicate 1 SD.

dissolved in 5 ml Aquasol. The radioactivity in the filterpads was determined with a Mark II liquid scintillation spectrometer (Nuclear Chicago Corp. Des Plaines, Il). Cell counts were determined in parallel cultures after trypsin treatment in a haemocytometer.

RESULTS

The SDS treated interferon preparation was fractionated on a Sephadex G-25 column, and eluted with distilled water in 3 ml fractions. Each fraction was tested on L cells for antiviral activity and effect on cell multiplication (Fig. 1). Fractions 6 and 7 contained the major part of AV activity and fractions 13 and 14 the CMI activity. The interferon titre of the AV pool (fractions 6 and 7) was 10000 units per ml and of the CMI pool (fractions 13 and 14) 25 units per ml.

Osteosarcoma cells were cultured in the presence of pooled AV or CMI fractions or interferon preparation. After 3 days' incubation the cell counts were determined. The data presented in Table 1

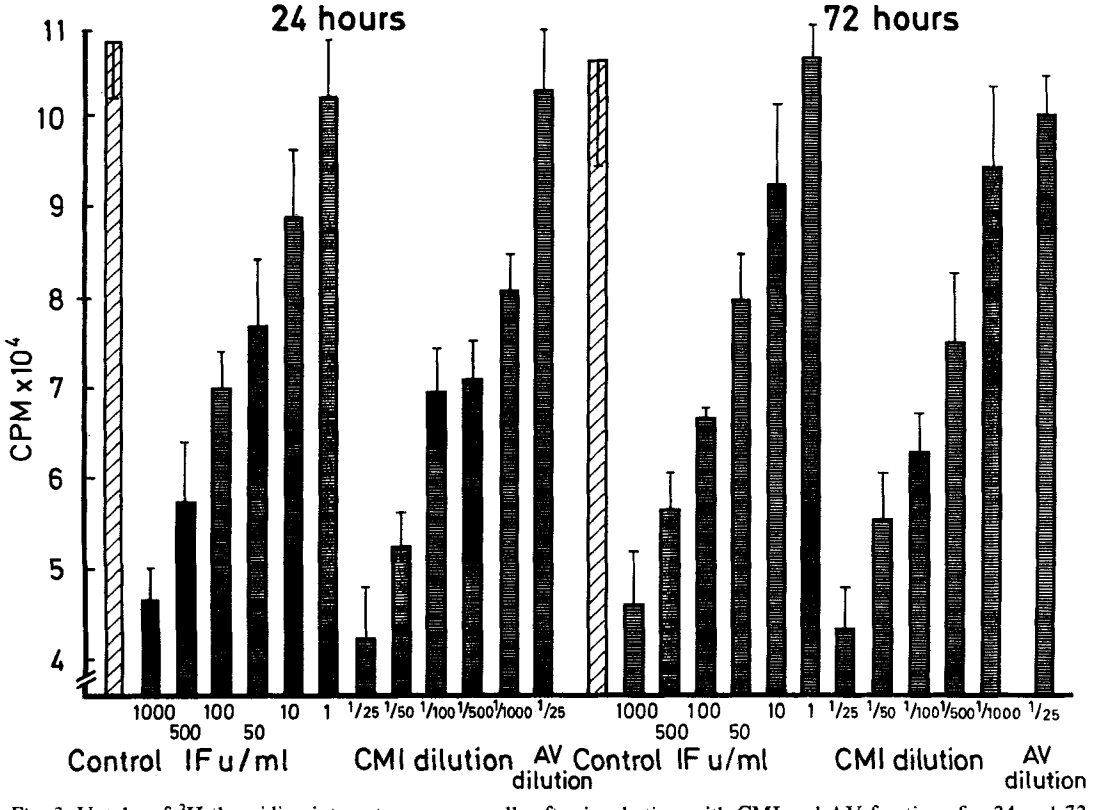

Fig. 3. Uptake of ^{3}H-thymidine into osteosarcoma cells after incubation with CMI and AV fractions for 24 and 72 hours. Vertical bars indicate 1 SD.

show that interferon and the CMI pool dose-dependently inhibited the growth of osteosarcoma cells. The AV pool had only a minor effect on the cell count. Although this effect seems dose-dependent the differences were not significant from the controls.

In the next experiments the effect of interferon preparation and the CMI fractions on the growth curve of osteosarcoma cells was examined. As shown on Fig. 2, the suppression by the two agents was comparable. From the fourth day of treatment the number of viable cells was significantly reduced by either agents compared to control cultures.

The growth-inhibitory effect could be visually shown, when osteosarcoma cells were grown with various concentrations of interferon preparations or AV or CMI fractions, and the cultures were stained after 7 days of incubation. Cell growth was clearly suppressed by 50 units of interferon and by comparable dilutions of CMI fractions (1:500). AV fractions had no visible effect on growth.

To further delineate the effect of CMI and AV fractions on osteosarcoma cells, the uptake of ^{3}H-TdR into the cells was determined following treatment with serial dilutions for 24 and 72 hours. Serial dilutions of interferon preparation were included for comparizon. At the end of the incubation the cultures were pulsed with 1 μCi/well of ^{3}H-TdR for 4 hours. The data in Fig. 3 show that interferon (10000 to 10 units per ml) and CMI fractions (1:25 to 1:1000 dilutions) dose-dependently reduced the uptake of ^{3}H-TdR. AV fractions had only a minor influence on the uptake at 1:25 dilution (containing 400 units of interferon per ml). The degree of inhibition was similar after 24 and 72 hours treatment. The viable counts were not significantly different after 24 hours. After 72 hours the number of cells was dose-dependently reduced by the interferon and by the CMI fractions up to 60 per cent of the controls.

DISCUSSION

The osteosarcoma cells from C57Bl/6 mice were established from a tumor produced by irradiation. Although the presence of C particles has been shown in these cells, the significance of these particles is not known. In many respects the murine osteosarcoma seems to be similar to the human osteosarcoma now being treated with interferon with promising results (18). Both systems are highly sensitive to treatment with homologous interferon *in vitro* (11, 19). Therefore this model seems to be well fitted for experimental studies with interferon. The present findings confirm the sensitivity of murine osteosarcoma cells to the growth-inhibitory activity of a homologous interferon preparation after up to 70 passages *in vitro*.

A physical separation of antiviral and cell multiplication inhibitory activities of partially purified human leukocyte and murine fibroblast interferon preparations has been reported from our laboratory earlier (4, 6). To confirm these observations we decided to test the active fractions on a different cell system highly sensitive to the cell multiplication inhibitory activity of interferon. Treatment of interferon preparation to separate the CMI activity was performed as reported earlier (4). The CMI and AV activities were demonstrated in the same fractions as previously found in L- cells. The low molecular weight fractions containing the bulk of CMI activity did not inhibit VSV multiplication in L cells. However, all antiviral fractions contained minor quantities of CMI activity.

In addition to reducing the cell count the fractions containing CMI activity also reduced the incorporation of ^{3}H-TdR into the cells, similarly to interferon effect (3). The uptake was not correlated to cell counts. However, after 24 hours treatment with interferon or CMI fractions the cell count was not significantly different from that of control cultures. The effect on TdR incorporation is therefore probably not only a function of the cell count but also inhibition of DNA synthesis or possibly a reduction of the transport of the radioactive compound through the cell membrane (1, 8, 9). Fractions containing AV activity showed only a minor and not significant effect on TdR incorporation.

The interferon preparation employed, although partially purified, still contained a high proportion of non-interferon substances. One possible explanation for the separation of activities, as proposed earlier (4, 6), may be that the interferon molecule carries both the AV and the CMI activities and that these are separated through the treatment by SDS at 100 °C. This hypothesis may not be true, since recently it has been reported that apparently homogeneous and pure preparation of mouse interferon, produced in a similar system as ours, still strongly inhibited cell growth (10).

An alternative explanation may be that the CMI activity is a contaminant, probably produced together with interferon as a common response to indecers. It has been shown (*M. Evinger,* Nutley, N. J. personal communication) that the proportionality of AV and CMI activities in different molecular species of apparently pure human leukocyte interferon may vary considerabely. Some fractions contained low CMI activity together with high AV activity, and vice versa. Our data are not contradic-

tory to these findings. Although the bulk of CMI activity was separated in the low molecular weight fractions a minor CMI activity was also associated with the AV fractions in most of our experiments. It is possible that the SDS-heat treatment selected interferon molecular species with low CMI activity. On the other hand the same treatment separated a low molecular weight CMI active component, not necessarily part of the interferon molecule.

Another alternative possibility is that the treatment caused formation of complexes with CMI effect. SDS alone is cell growth inhibitory. Presence of free SDS in the CMI fraction has been excluded earlier (4), but it is possible that the employed method did not detect the hypothetic complexes. Against this hypothesis argues also the finding of seemingly identical CMI fractions (6) separated from interferon preparation by a different method without presence of SDS.

To solve this question it is mandatory that similar separation attempts should be done on pure interferon when sufficient quantities will be available.

Parts of the experimental work have been carried out at the Department of Pediatrics, Infectious Diseases Unit, University of Utah, Medical Center, Salt Lake City, Utah.

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