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SELECTIVE INHIBITION BY INTERFERON OF SERUM-STIMULATED BIOCHEMICAL EVENTS IN 3T3 CELLS

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SUMMARY

We examined the effect of mouse interferon on the stimulation of [$^{86}Rb^+$] uridine, 2-deoxyglucose and Pi uptake and of ornithine decarboxylase activity produced by serum in quiescent cultures of Swiss 3T3 cells. We found that interferon causes a differential dose-dependent inhibition of the stimulation of ornithine decarboxylase activity and the second phase of Pi uptake. Other protein-synthesis independent or dependent events are not affected.

While interferons inhibit the proliferation of a variety of cells in vitro and in vivo, the basis of the antiproliferative effect remains unknown (1-7). Recent findings indicate that both G1 and S+G2 phases of the cell cycle are extended by interferon in normal (7) and malignant cells (6). The effect of adding interferon to quiescent 3T3 cells stimulated by serum to leave G_1/G_0 and enter DNA synthesis, is to lengthen G_1 and to reduce their rate of entry into S phase (7).

Prior to the onset of DNA synthesis, serum added to cultures of fibroblasts resting in G_1/G_0 stimulates a complex array of biochemical events. Within minutes of serum addition, there is an increase in the uptake of [$^{86}Rb^+$] (8), uridine (9,10), 2-deoxyglucose (11) and Pi (12). These early events are not dependent on continuous protein synthesis (13). After several hours of serum-stimulation, there is a further rise in the uptake of 2-deoxyglucose and Pi (13) and a striking elevation in the activity of ornithine decarboxylase (EC4.1.1.17), a key enzyme in polyamine synthesis (14). These late events are completely blocked by inhibitors of protein synthesis (13).

To gain further insight into the anticellular action of interferon, we examined whether any of these pre-replicative events stimulated by serum are affected by interferon. We found that interferon exerts a remarkable and differential inhibition on the above events.

MATERIALS AND METHODS

Cell culture. Swiss 3T3 mouse cells were grown to confluence in 30 or 90mm Nunc Petri dishes in Dulbecco's modified Eagle's medium, 10% fetal calf serum (FCS),

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100 units/ml penicillin and 100µg/ml streptomycin in a humidified atmosphere of 10% CO_2 and 90% air at 37°C. To ensure that confluent cultures were arrested in G_0 they were incubated for 24 hr at 37° with fresh medium containing 10% medium from the confluent cultures (final serum concentration 1%).

Interferon. Preparations of mouse L cell interferon with specific activity of 5×10^5 reference units (U)/mg of protein (M. Johnson, Wellcome) or 2×10^7 U/mg of protein (R.M. Friedman, National Institutes of Health) were used. The human interferon used was Namalva lymphoblastoid cell interferon with a specific activity of 3×10^7 U/mg of protein (K. Fantes, Wellcome).

Uptake measurements. The procedures for measurement of uptake of [^{3}H] uridine (10) or [$^{86}Rb^+$] (8) into acid soluble cellular pools were similar to those described previously. To measure uptake of deoxyglucose, duplicate 30mm plates were preincubated for 30 min in glucose-free medium before adding 2-[^{3}H]-deoxyglucose (0.2mM 2.5µCi/ml) in 1.5ml of medium; after 10 min acid soluble radioactivity was determined (11). When cultures were to be used for measuring uptake of Pi they were washed with phosphate-free medium before adding dialyzed serum (in phosphate-free medium). At the times indicated, [^{32}Pi] (0.1mM 2.5µCi/ml) was added to duplicate cultures (30mm dishes) and acid-soluble radioactivity estimated after 10 min (12).

Assay of ornithine decarboxylase. The procedure used was similar to that reported by Hogan (15). For each assay 200µl of extract was prepared from three 90mm culture dishes ($1\text{-}2 \times 10^7$ cells). The final concentration of the various components in the enzyme assay was 0.1mM EDTA, 0.05mM pyridoxal phosphate, 5mM dithiothreitol, [^{14}C]-ornithine (0.2µCi, 0.2mM) and 50mM of HEPES pH 7.1. The release of radioactive CO_2 as measured above was linear for at least 60 min of incubation at 37° and was also proportional to the amount of cell extract used. (The amount of [^{14}C]-carbon dioxide released did not vary more than 10% in duplicate samples and the values presented are the average of duplicate determinations.) To ensure that the release of [^{14}C]-carbon dioxide from 1-[^{14}C]-ornithine was a valid measure of enzymatic activity, the formation of [^{3}H]-putrescine from 5-[^{3}H]-ornithine was determined. The labeled putrescine was isolated from the reaction mixture and analyzed using the method of Inoue and Mizutani (16). The amount of [^{3}H]-putrescine formed was proportional to the amount of [^{14}C]-carbon dioxide released.

Other materials and methods. For determination of DNA synthesis the cultures (30mm dishes) were exposed to methyl-[^{3}H]-thymidine (4µCi/ml, 0.2µM) for 30 hr after serum stimulation and the incorporation into acid-precipitable DNA was measured as described previously (7). Protein was determined by the method of Lowry et al. (17) using saline washed cells or homogenates. All radioactively labeled chemicals were obtained from the Radiochemical Centre. Other chemicals used were purchased from Sigma Chemical Co.

RESULTS AND DISCUSSION

The early activation by serum of the uptake of [$^{86}Rb^+$], uridine, 2-deoxyglucose, and Pi in quiescent cultures of 3T3 cells was unaffected by interferon whether added concomitant with or 12 hours before serum. On the other hand, interferon exerted a selective inhibitory effect on the late responses elicited by serum. Figure 1A shows that serum addition causes a gradual increase in ornithine decarboxylase activity which reached a maximum (30-fold increase) within 8 hours, and which is prevented by cycloheximide. In cultures stimulated with serum in the presence of interferon, there

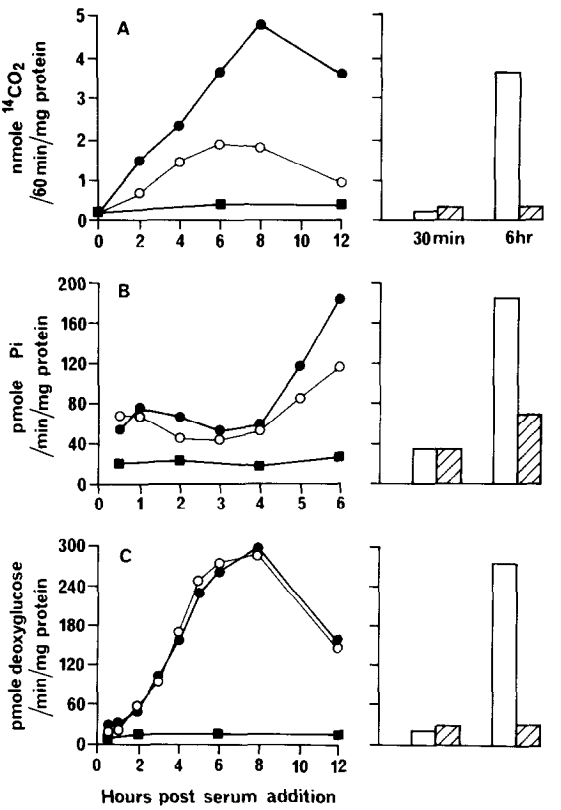

Fig. 1. The effect of interferon on A) the induction of ornithine decarboxylase activity, B) uptake of ^{32}Pi and C) uptake of 2-[^{3}H]-deoxyglucose in quiescent 3T3 cells stimulated with serum. Quiescent cultures (30mm or 90mm dishes) were prepared as described in Materials and Methods and medium changed to either fresh medium without serum ■—■, medium with 10% dialyzed FCS ●—● or medium containing 10% dialyzed FCS and 10^4 U/ml of mouse interferon ○—○. At the times indicated, the level of ornithine decarboxylase (A), the uptake of Pi (B) or the upake of 2-[^{3}H]-deoxyglucose (C) were measured as described in the Methods section. The bar graphs presented in A, B and C represent the level of the respective activity at 30 min or at 6 hr following serum stimulation in cultures incubated in 10% dialyzed FCS, , or 10% FCS + 10ug/ml cycloheximide, ▨.

was a significant reduction in ornithine decarboxylase activity. Interferon also inhibited the second, cycloheximide-sensitive phase of increased Pi uptake (Fig. 1B). In sharp contrast, interferon had no effect on the large stimulation of 2-deoxyglucose uptake produced by the serum (Fig. 1C). The results show a marked differential effect of interferon on protein synthesis-dependent events.

The differential inhibitory effect of interferon was further substantiated when its action was assessed at different concentrations added to quiescent cultures simultaneously with fresh serum. The results presented in Fig. 2 show that interferon causes a potent, dose-dependent inhibition of the induction of ornithine decarboxylase activity, of the second phase of Pi uptake and of the synthesis of DNA in serum-stimulated 3T3

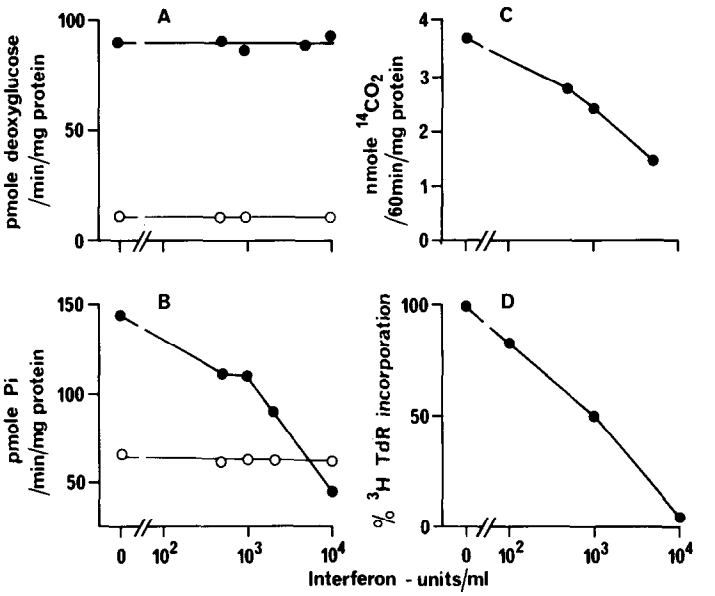

Fig. 2. Effect of different doses of interferon on (A) the uptake of 2-[^{3}H]-deoxyglucose, (B) the uptake of ^{32}Pi, (C) the induction of ornithine decarboxylase activity and (D) synthesis of DNA in quiescent 3T3 cells stimulated with serum. Quiescent cells were stimulated with dialyzed FCS (10%) with or without varying doses of mouse interferon. The uptake of 2-[^{3}H]-deoxyglucose and ^{32}Pi into cells was measured at 30 min (o—o) and 6 hr (●—●) following serum stimulation. The ornithine decarboxylase activity contained in the cultures was measured at the end of 6 hours following serum stimulation (see Methods section). Values for DNA synthesis are expressed as percent of the control. Quiescent and serum-stimulated cultures incorporated 5×10^{3} and 4.3×10^{5} c.p.m./culture during 30 hours incubation with [^{3}H]-thymidine. Interferon specific activities: A.B. and D. 2×10^{7} U/mg; C. 5×10^{5} U/mg.

cells. These events are inhibited by the same range of interferon concentration while even at the highest dose used (10^{4} U/ml) interferon had no effect on the early or late phase of activation of [^{3}H]-deoxyglucose uptake into cells.

Two observations suggest that the inhibitory effects described above are indeed mediated by mouse interferon. Firstly they were not obtained when human lymphoblastoid interferon was used in place of mouse interferon. Secondly, comparable degrees of inhibition were obtained using a different preparation of interferon with a 40-fold higher specific activity suggesting that the effects observed are interferon-specific.

The induction of ornithine decarboxylase in both eukaryotic and prokaryotic cells is modulated by a protein, the ornithine decarboxylase antizyme, which inhibits the enzyme non-competitively (18,19). The results shown in Table 1 indicate that extract originating from the interferon-treated cultures possesses no significant inhibitory effect on the enzymatic activity present in untreated cultures. This suggests that the

Table 1. Absence of ornithine decarboxylase inhibitory substance(s) in interferon-treated cultures of 3T3 cells.

EXTRACTS PREPARED FROM	ENZYME ACTIVITY (nmoles/60min/mg. protein)	
	Found	Expected
1. Cells stimulated with 10% dialyzed FCS	2.8	
2. Cells stimulated with 10% dialyzed FCS + interferon	0.5	
3. 1 + 2 (1:1)	2.9	3.3
4. 1 + 2 (1:1) and preincubated	3.0	3.3

Six hours after the indicated addition to cells, extracts were prepared (1) from the cells stimulated with 10% dialyzed FCS and (2) from cells stimulated with 10% dialyzed FCS + 10^4U/ml interferon. These extracts were assayed individually or in combination. In sample 3 equal volumes of extract from (1) and (2) were mixed and assayed immediately, while in sample 4 the mixture was incubated for 15 min at 37° before the assay.

observed inhibition of enzymatic activity by interferon was not mediated by the presence of an antizyme in cell extracts.

Recently, Yau et al. (20) reported that inhibitors of elongation steps in protein synthesis (e.g. cycloheximide) mimic interferon treatment in that they selectively inhibit the synthesis of certain viral proteins. The experiment presented in Fig. 3 was performed to determine whether low concentrations of cycloheximide exert similar discriminatory effects on the serum-stimulated events examined here. Cycloheximide, at the low doses used, did not reproduce the differential effect of interferon; the relative degree of inhibition of stimulated deoxyglucose uptake or induction of ornithine decarboxylase activity was not significantly different (Fig. 3). Furthermore we have not detected any consistent inhibition by interferon of overall protein synthesis measured up to 12 hours after its addition (data not shown). These findings indicate that the selective effect of interferon on protein synthesis-dependent events which are stimulated by serum does not result from a small inhibition of overall protein synthesis as is produced by low concentrations of cycloheximide.

The findings presented here have several important implications. The differential inhibition of serum-stimulated events produced by interferon provides a powerful tool for elucidating which events are essential for the progression of the cells through G_1, for identifying which proteins are critically involved in the initiation of DNA synthesis, and for furthering studies on the anticellular action of interferon. The selective inhibition of the stimulation of ornithine decarboxylase activity by interferon

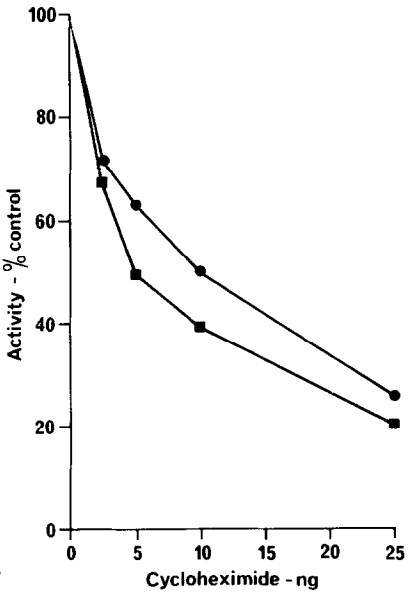

Fig. 3. Effect of different doses of cycloheximide (ng/ml) on the uptake of 2-[^{3}H]-deoxyglucose and induction of ornithine decarboxylase activity in quiescent 3T3 cells. Quiescent cells were exposed to 10% dialyzed FCS and varying doses of cycloheximide. The activity present in the cultures at 6 hr after serum stimulation was determined as described in Methods. The values presented are expressed as per cent of that obtained in cultures stimulated with serum in the absence of the inhibitor. ●—● uptake of 2-[^{3}H]-deoxyglucose, ■—■ ornithine decarboxylase activity.

is of particular interest because this enzyme has been implicated in the mechanism of action of tumor promotors (21) and because interferon has proved to be a potent anti-tumor agent (22). The possibility that the antitumor activity of interferon can in part be attributed to a sustained inhibition of ornithine decarboxylase activity warrants further experimental work.

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