ous constituents of the cells, at least during the early stages of endogenous respiration and before death becomes a major factor. The loss of nitrogen from the cells as NH_3 would result in some shift in internal conditions. The results indicate that the oxidative assimilatory and endogenous respiratory activities of the oxidative yeast *R. graminis* are similar to those reported for fermentative yeasts and for bacteria. The results of the RQ studies and those on the amino acid pool and on NH_3 production indicate that amino acids are a major endogenous substrate for this organism. Carbohydrate, which is an endogenous substrate for various species of yeast, does not appear to act as such to a marked extent for *R. graminis*.

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The Effect of Interferon on Focus Formation and Yield of Murine Sarcoma Virus *in Vitro* (33698)

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The available evidence supports the view that interferon plays a significant role in host-resistance to primary viral infections (1). Moreover, various oncogenic viruses recently were shown to be susceptible to the inhibitory action of interferon (2–6). With the demonstration of a viral agent inducing sarcomas in mice (7, 8) and the development of an *in vitro* assay system for this virus (9), it has become possible to test the susceptibility of this murine sarcoma virus (MSV) to the inhibitory action of interferon. The data presented below indicate that mouse serum interferon induced by Newcastle disease virus *in vivo* does interfere with focus formation and yield of MSV *in vitro*.

Materials and Methods. A cell-free pool of murine sarcoma virus (MSV) derived from sarcomas in Balb/c mice was kindly provided by J. Moloney, NCI, NIH, and was used as test material in all these experiments. The titer of this virus was $10^{7.6}$ pfu/ml. There was a direct relationship between dilutions of this virus and the focus count. The Herts strain of Newcastle disease virus (NDV) was obtained through the courtesy of S. Baron, NIAID, NIH. It was propagated in embryonated chicken eggs and had a titer of $10^{9.3}$ pfu/ml. Vesicular stomatitis virus (VSV) was obtained from Y. Hirshaut, NCI, NIH; it was propagated in mouse embryo fibroblast cultures and had a titer of $10^{7.0}$ pfu/ml. Concentrated plasma-derived Rauscher leukemia virus (RLV) was obtained from Mason Research Institute and demonstrated a titer of between 10^6 and 10^7 $TCID_{50}$/ml by means of an interference assay using MSV as challenge virus (10).

Mouse embryo fibroblast (MEF) cell cultures and chick embryo fibroblast (CEF) cell cultures were prepared from 9–14-day-old embryos by trypsinization. Medium for these cultures consisted of Eagle's minimum essential medium (MEM) supplemented with glutamine and with 10% (for growth) or 2% (for maintenance) heat-inactivated fetal calf serum (Hyland). The JLS-V9 cells, a cell line derived from mouse bone marrow (11), were grown in McCoy's 5A medium supple-

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mented with glutamine and with 20% (for growth) or 2% (for maintenance) heat-inactivated fetal calf serum. Penicillin and streptomycin were included at concentrations of 100 units/ml and 0.1 mg/ml, respectively, in all media.

Plaque assays for NDV and for VSV, as well as interferon assays using VSV as challenge virus, were performed as described earlier (12). Focus formation assays for MSV were performed by inoculating non-confluent cultures with dilutions of MSV, incubating for 2 hr at 37°, and adding fresh growth medium. Fluids were changed at 2–3 days, and foci were counted microscopically at 4–6 days. In titrating pools of tissue culture-derived MSV, as in determination of yield of MSV, *in vitro*, replicate cultures were inoculated with 0.1 ml of an MSV dilution and 0.1 ml of a 1:10 dilution of helper virus (RLV) in order to measure total virus yield.

The interferon used was induced in NIH general purpose adult Swiss mice by intravenous inoculation with 0.4 ml of undiluted NDV via the lateral tail veins. Control serum was obtained from similar mice inoculated i.v. with the same volume of allontoic fluids from NDV-free chick embryos. The blood was collected by incision of the axillary blood vessels 6 hr later and stored overnight at 4°. The clot was removed the next day and the serum was clarified by centrifugation in a PR 2 International centrifuge at 1500 rpm for 15 min. To eliminate residual NDV, the serum was then treated with concentrated HCl to give a pH of 2, refrigerated 5 days, and then neutralized with NaOH. It was then centrifuged at 100,000*g* for 1 hr in a Spinco model L-2 ultracentrifuge. The supernatant fluids were stored undiluted at −20° until used.

This preparation was assayed for biological activity against VSV and had an inhibitory titer of 10^4 PDD_{50}/ml (13). It was also tested and found to possess the following properties of interferon: cell species specificity, nonsedimentability at 100,000*g*, inability to inactivate virus directly, inhibitory activity against heterologous virus, and insensitivity to acid treatment. Furthermore, to determine whether acid treatment had indeed eliminated all interference due to any residual NDV in the serum, the effect of acid treatment on the capacity of NDV to interfere with MSV focus formation was tested. Cultures were inoculated with MSV and either growth medium alone or growth medium containing NDV, the latter either untreated or treated with acid for 1 or 7 days. Acid treatment of NDV for both 1 and 7 days prevented the inhibitory activity of NDV on MSV focus formation.

TABLE I. Relation of MSV Focus Formation to Concentration of Interferon.

Treatment	No. of foci	Av	Inhibition (%)
None	177, 166, 153, 203	175	—
Control mouse serum			
1:10	179, 183	181	0
1:20	141, 144	143	18
1:40	189, 160	175	0
Mouse interferon serum			
1:10	0, 0, 1, 0	0.25	100
1:20	1, 4, 2, 1	2	99
1:40	12, 6, 7, 8	8	95
1:80	33, 32, 24, 22	28	84
1:160	45, 52, 57, 49	51	71
1:320	72, 75, 73, 88	77	56

Results. Effect on focus formation. Non-confluent, replicate cultures of JLS-V9 cells were inoculated simultaneously with 0.2 ml of MSV (about 200 ffu) and 0.3 ml of either growth medium, dilutions of control mouse serum or dilutions of mouse interferon serum. The plates were incubated for 1 hr at 37° and then growth medium was added. The plates were fluid changed on day 2 and foci were counted on day 4 (Table I). The results indicated a direct relationship between inhibition of focus formation and the concentration of interferon. The slight decrease in number of foci observed in the plates treated with a 1:20 dilution of normal mouse serum was probably not significant. In any case, the observation of a more than 50% decrease in number of foci in the plates treated with a dilution of interferon serum of 1:320 when

TABLE II. The Relation of MSV Focus Formation to the Concentration of Interferon under Different Conditions of Treatment.

Treatment	Dilution	16 hr prior to MSV adsorption		Continuously after MSV adsorption		Simultaneously with MSV adsorption	
		No. of foci	Inhibition (%)	No. of foci	Inhibition (%)	No. of foci	Inhibition (%)
None	—	145, 132 155, 149	—	116, 132 123	—	58, 41 75, 68	—
Control mouse serum	1:10	135, 174	0	73, 75	40	73, 91	0
	1:32	146, 152	0	124, 125	0	105, 81	0
Mouse interferon serum	1:10	65, 72	53	8, 10	93	ND	ND
	1:32	63, 72	53	24, 26	81	ND	ND
	1:100	72, 76	49	40, 46	65	4, 7	90
	1:320	75, 80	47	52, 54	57	24, 25	59
	1:1000	68, 74	51	62, 64	49	38, 49	28
	1:3200	ND	ND	ND	ND	54, 65	1
	1:10,000	ND	ND	ND	ND	61, 83	0

none was observed in plates treated with control mouse serum at dilutions of 1:10 and 1:40 suggests strongly that the inhibition observed in the interferon-treated cultures was not due to nonspecific serum inhibitors nor to a cytotoxic effect of the serum.

Effect of various methods of treatment. Various methods of treating the cells with interferon were tested: (a) treatment prior to MSV adsorption, (b) treatment following MSV adsorption, and (c) treatment simultaneously with MSV adsorption. In part (a), duplicate, nonconfluent JLS-V9 cell cultures were treated with 3.2 ml of growth medium, dilutions of control mouse serum, and dilutions of mouse interferon serum in growth medium. The cultures were incubated at 37° for 18 hr. The plates were then washed 5 times to remove excess interferon, 0.2 ml of MSV (about 150 ffu) were added, and the plates were incubated for 1 hr at 37°. Growth medium was then added. Fluids were changed on day 2, and foci were counted on day 4. In part (b), nonconfluent JLS-V9 cell cultures were inoculated with 0.2 ml of MSV (about 125 ffu), incubated for 1 hr at 37°, and then washed 5× to remove excess, unadsorbed virus. Duplicate plates were then treated with 3.2 ml of growth medium, dilutions of control mouse serum, or dilutions of mouse interferon serum. The cultures were incubated at 37°, the fluids were changed on day 2, and foci were counted on day 4. In part (c), duplicate, nonconfluent JLS-V9 cell cultures were inoculated simultaneously with 0.2 ml of MSV (about 60 ffu) and 0.3 ml of growth medium, dilutions of control mouse serum and dilutions of mouse serum interferon. The cultures were incubated at 37° for 1 hr, at which time growth medium was added. Fluids were changed at day 2, and foci were read at day 4 (Table II). The results indicated that the inhibition of focus formation due to exposure to interferon before MSV was not related to the dilution of interferon applied (part a). A direct relationship between inhibition of focus formation and concentration of interferon was observed only when interferon was present in the fluids throughout the period of focus formation (parts b and c).

The loss of interferon activity with time. To determine the length of time that cells treated with interferon remained resistant to MSV focus formation, the following experiment was performed: replicate cultures of JLS-V9 cells were treated with either growth medium or mouse serum interferon (about

TABLE III. The Loss of Interferon Activity with Time.

Time of MSV adsorption relative to IF treatment	Treatment	No. of foci	Inhibition (%)
Immediately after	None	84, 90, 83	—
	Interferon	32, 40, 37	58
24 hr later	None	17, 12, 30, 17	—
	Interferon	35, 16, 19	0

500 PDD_{50}) for 3 hr at 37°. The cultures were washed five times. Interferon-treated and untreated cultures were then inoculated with 0.2 ml MSV (about 100 ffu) either immediately after or 24 hr later. Growth medium was added after an adsorption period of 1 hr. Fluids were changed on day 2 and foci were counted on day 4 (Table III). The results indicated that cells treated with interferon were no longer protected against MSV 24 hr after treatment. The reduction in number of foci observed in control cultures inoculated 24 hr after interferon treatment occurred in their companion cultures can be explained by the fact that MSV focus formation is commonly reduced in more confluent cultures.

Effect on yield of MSV. Furthermore, replicate, nonconfluent cultures of JLS-V9 cells were treated with growth medium, 1:32 dilution of control mouse serum or 1:32 dilution of mouse interferon serum (about 320 PDD_{50}) for 16 hr at 37°. The cultures were then washed 10 times to remove excess proteins. Each culture was then inoculated with 0.1 ml MSV at an input virus to cell ratio of about 2. The cultures were incubated for 2 hr at 37° and then washed five times. Growth medium was added. Fluids from groups of three cultures of each type were collected and pooled at daily intervals for 4 days. These fluids were then titrated for MSV in JLS-V9 cell cultures with added Rauscher leukemia virus as helper (Fig. 1). Prior treatment of the cells with control mouse serum had no effect on the yield of MSV *in vitro*. Interferon treatment, however, reduced the yield of MSV significantly at 24 hr, but the effect was gradually overcome.

Finally, groups of three nonconfluent cultures of JLS-V9 cells were each treated with growth medium, dilutions of control mouse serum or dilutions of mouse interferon serum for 16 hr. At the end of this time, the cultures were washed three times and then inoculated with 0.2 ml of MSV at an input virus to cell ratio of about 2. The cultures were incubated 2 hr at 37° and then washed three times. Growth medium was then added and the cultures incubated at 37° for 24 hr. At this time, the fluids were collected from each group of three and pooled, centrifuged to remove cells, and titrated for MSV activity (Table IV). This experiment was repeated. In both instances, there was a direct relationship between inhibition of MSV yield and concentration of interferon. Although control mouse serum-treated cultures sometimes demonstrated slightly reduced yields of MSV

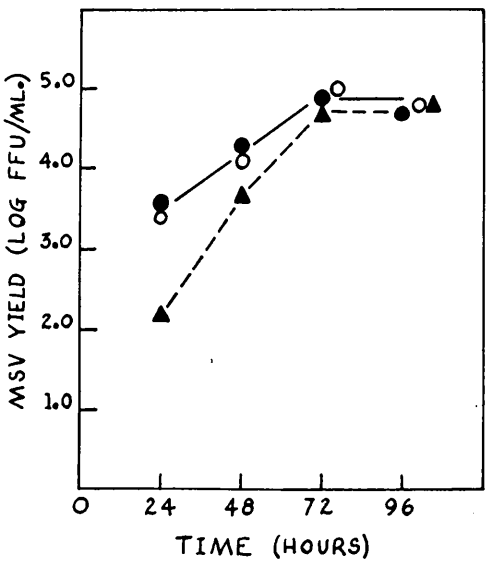

FIG. 1. The effect of interferon treatment on MSV yield *in vitro* with time: controls untreated, (●); controls treated with normal mouse serum, (○); yield from interferon treated cultures, (▲).

TABLE IV. Relation of 24-hr Yield of MSV to Concentration of Interferon.

Treatment	Dilution	Expt. 1 log titer/ml	Expt. 1 Reduction (%)	Expt. 2 log titer/ml	Expt. 2 Reduction (%)
Growth medium only		2.9	—	2.4	—
Control mouse serum	1:1000	2.9	0	ND	ND
	1:100	2.7	23	2.3	12.5
Mouse interferon serum	1:32,000	2.9	0	ND	ND
	1:10,000	2.8	15	2.3	12.5
	1:3200	2.6	45	2.1	42
	1:1000	2.4	68	1.8	71
	1:320	2.0	90	1.5	88
	1:100	1.6	95	1.1	95

(always less than 50%), only the interferon-treated cultures indicated significant reductions in yield.

Discussion. These results indicated that the treatment of mouse cells with mouse interferon serum protected the cells against focus formation by MSV and significantly reduced the 24-hr yield of MSV. Control mouse serum in comparable dilutions had no such effect. Nevertheless, the following tests were performed to establish more conclusively that the anti-MSV activity observed was indeed due specifically to the interferon present in the serum: (a) incubation of mouse serum interferon with MSV for 1 hr at 37° had no effect on the titration pattern of the MSV, indicating that the anti-MSV principle was cell-mediated and did not inactivate MSV directly; (b) centrifugation of mouse serum interferon at 100,000*g* for 3 hr had no effect on the anti-MSV potency of the preparation, indicating that the active inhibitory principle was not particulate; (c) incubation of mouse serum interferon for 1 hr at 37° with 0.06% trypsin completely abolished the anti-MSV activity of the preparation, indicating that the anti-MSV principle was digestible by a proteolytic enzyme; and (d) simultaneous testing of mouse serum interferon for inhibition of MSV forcus formation in JLS-V9 cells and in an established rat kidney cell line (NCI-NR cells) graciously donated by H. Duc-Nguyen, NCI, NIH (14), resulted in significant reduction in number of foci produced in mouse cells, but no measurable effect on focus number in rat cells, indicating that the anti-MSV activity was species specific. These results together with the fact that the anti-MSV activity was acid resistant suggest strongly that the inhibitory effect observed in the data presented was due specifically to the interferon present in the serum.

In general, on the basis of the relative potency of a single interferon preparation, VSV appeared to be at least 10 times as sensitive to interferon treatment as MSV. However, this may reflect the longer period of time required for focus formation and the reduced efficiency of interferon treatment with time rather than inherent reduced sensitivity. The development of a one-step growth cycle for MSV would provide the necessary basis for an ideal comparative study of the relative sensitivities of the two viruses to interferon.

The loss of the protective effect of interferon on cells with time was shown (a) by the loss of a direct relationship between inhibition of focus formation and concentration of interferon when the treatment was given prior to MSV adsorption and excess interferon washed away, due probably to the requirement of 4–6 days for focus formation because of the necessity for virus replication and reinfection of cells (9; Duc-Nguyen, personal communication); (b) by the direct observation of the loss of inhibition of focus formation when MSV was added 24 hr after interferon treatment; and (c) by the observation that the inhibitory action of interferon

on MSV yield *in vitro* was gradually overcome with time. Continuous treatment of the cells with interferon was therefore necessary to observe its inhibitory effect on focus formation. Similar results have been obtained by P. Sarma, NCI, NIH, working independently on the same general problem (personal communication). This temporal relationship between interferon treatment and MSV sensitivity may shed light on the difficulty in the past to demonstrate an effect of interferon treatment on murine leukemia virus-induced disease (15), and the need for frequent, high doses of interferon to demonstrate any such effect at all (16, 17).

It still remains to be shown whether MSV can induce interferon formation, and whether interferon inhibits MSV replication alone, leukemia virus replication alone, or both.

Summary. Interferon-treated cells yielded less murine sarcoma virus and were less susceptible to focus formation than were untreated cells or cells treated with control mouse serum. The protective effect of interferon appeared to be lost or at least significantly reduced 24 hr after treatment.

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