Interferon Synthesis in X-Irradiated Animals
III. The High Radiosensitivity of Myxovirus-Induced Circulating Interferon Production (33799)

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Production of circulating interferon (1, 2) upon stimulation with Sindbis or Newcastle disease virus is markedly depressed in C_3H mice following a 1000 R total-body X-irradiation (3). This inhibition of interferon synthesis can be ascribed to an impairment of hematopoietic function and can be reversed by grafting syngeneic or even xenogeneic bone marrow cells into lethally irradiated recipients (4). We have furthermore found that the circulating interferon response to Newcastle disease virus was strongly reduced by a dose of X-rays as low as 125 R, while under similar conditions the interferon response to Sindbis virus remained unaltered (5). This suggested the existence of circulating interferon producing systems with distinctive radiosensitivities, and it was therefore relevant to pursue this problem and investigate the effect of X-irradiation on circulating interferon response to several viruses.

Materials and Methods. (1) Animals used throughout the experiments were 6–8-week-old male C_3H/He mice. Only first and second generation progeny derived from breeding stock purchased from the Laboratory Animals Centre (M.R.C., Carshalton, Surrey, U. K.) were used.

(2) *Virus strains.* Origin, titer, and method of preparation of viruses used as interferon inducers are given in Table I.

(3) *Tissue culture media.* Culture medium of L cells was Eagle's minimum essential medium (6), prepared from powdered medium (Gibco, Long Island, New York; 8% inactivated calf serum was added for growth, and 3% of the same serum for maintenance.

For cultures of mouse macrophages, we made use of medium 199, obtained from the Pasteur Institute (Garches, France) and enriched with 20% inactivated calf serum.

(4) *Preparation of macrophage cultures.* To harvest peritoneal cells, mice were killed by cervical dislocation, the skin over the peritoneum was reflected, and 5 ml of medium 199 enriched with 20% calf serum was injected into the peritoneal cavity. After gentle abdominal massage, as much fluid as possible was withdrawn with a syringe and collected in a vessel immersed in an ice bath.

The fluids obtained from all mice were pooled and kept chilled until distribution into small plastic petri dishes of 3-cm diameter. Five ml of the peritoneal cell suspension was added to each petri dish, at a concentration of 600,000 nucleated cells/ml. After overnight incubation, the cultures were shaken to resuspend nonattached cells and the culture fluids were aspirated. This procedure was repeated once, and finally 5 ml of fresh culture medium was added. About 30% of the cells remained sticking to the bottom of the culture vessel; these cells were considered to be macrophages (7).

(5) *Induction of circulating interferon in mice.* A 0.2-ml aliquot of virus suspension was injected into the right orbital sinus.Six to 8 hr later, blood was drawn from an orbital sinus; samples were left to coagulate overnight in the refrigerator and sera were then separated by 2 min centrifugation at 15,000*g*. Serum pools were made by taking the same amount of serum, generally 0.1 ml, from each of the six animals that made up an experimental group. Serum pools were diluted tenfold in Eagle's medium, dialyzed for 48 hr against Sörensen buffer at pH 2 and for another 48 hr against Eagle's medium.

(6) *Titration of interferon.* Interferon was measured by a plaque reduction method (8) in L cell cultures with vesicular stomatitis virus as challenge. Interferon titers are expressed as units, one unit corresponding to the amount of inhibitor necessary to reduce

TABLE I. Viruses Used as Interferon Inducers.

Strain and origin	Titer of virus used to induce interferon	Method of cultivation
Newcastle disease virus (Kumarov strain) received in 1962 from Dr. C. Huygelen RIT-Genval, Belgium	10^8 egg ID_{50}/0.1 ml	Chorioallantoic cavity of chick embryo
Influenza A Sing isolated in Belgium in 1957	10^8 egg ID_{50}/0.1 ml	Chorioallantoic cavity of chick embryo
Parainfluenza 1 (Sendai) received in 1966 from Dr. I. Gresser Villejuif, France	$10^{7.5}$ egg ID_{50}/0.1 ml	Chorioallantoic cavity of chick embryo
Mumps received in 1968 from Dr. I. Gresser Villejuif, France	500 HU/0.5 ml	Chorioallantoic cavity of chick embryo
Sindbis (Egypt AR 339) received in 1961 from Dr. P. Y. Cheng Rockefeller Foundation	10^5 pfu/0.1 ml (in L cells) $10^{8.5}$ egg ID_{50}/0.1 ml	Chorioallantoic cavity of chick embryo or tissue culture of chick embryo cells
Semliki Forest (Kumba) received in 1965 from Dr. J. Sonnabend Mill Hill, London	5×10^6 pfu/0.1 ml (in L cells)	Chick embryo fibroblast tissue culture
Encephalomyocarditis (EMC-2) received in 1965 from Dr. J. Sonnabend Mill Hill, London	5×10^7 pfu/0.1 ml (in L cells)	Mouse L cell tissue culture
Vesicular Stomatitis (Indiana) received in 1965 from Dr. I. Gresser Villejuif, France	6×10^6 pfu/0.1 ml (in L cells)	Chick embryo fibroblast tissue culture
Vaccinia received in 1965 from Dr. J. Sonnabend Mill Hill, London	2×10^8 egg ID_{50}/0.1 ml	Chick embryo fibroblast tissue culture

the number of challenge virus plaques by 50%. A more detailed description of our titration method can be found in a previous paper (3).

The viral activity measured in the sera of mice after intravenous injection of the viruses tested was attributed to interferon(s), using criteria published previously (4).

(7) X-irradiation. All irradiations were performed with a 225 kV X-ray apparatus (11 mA, 225 kV, filtration 0.5 mm Cu, half value layer 1.5 mm Cu). More details can be found in a previous paper (3).

Experimental Results. *Effect of irradiation on circulating interferon induction.* Previously published results have suggested the existence of different systems of interferon induction for Sindbis and for NDV (3). Since then, we have observed that the procedure of following interferon induction on successive days after exposure of mice to 1000 R gives variable results; generally, the maximal inhibition of Sindbis induced interferon appears earlier than maximal inhibition of NDV induced interferon, but the latter is often already quite reduced 24 hr after irradiation. More consistent results can be obtained by studying interferon induction in different groups of mice, exposed to varied amounts of radiation, and injected with virus 4 days later. This method has provided additional evidence for the involvement of different circulating interferon producing systems for Sindbis virus and for NDV (5). The implications of these findings however were of limited value since only two viruses had been

TABLE II. Effect of Varying Doses of X-Irradiation on the Induction of Serum Interferon by Different Viruses (4 days after irradiation).

Dose of X-rays in roentgen[a]	NDV		Influenza A		Sendai		Mumps
	Expt. 1	Expt. 2[d]	Expt. 1	Expt. 2	Expt. 1	Expt. 2	
0	2900[b]	5000	760	230	1100	1480	400
125	115 (4)[c]	1400 (28)	120 (16)	25 (11)	290 (26)	380 (26)	90 (22)
250	58 (2)	500 (10)	32 (4)	10 (4,5)	105 (10)	165 (11)	16 (4)
500	26 (1)	440 (9)	16 (2)	10 (4,5)	105 (10)	230 (16)	22 (6)
1000	26 (1)	550 (10)	16 (2)	10 (4,5)	145 (13)	250 (17)	26 (7)

	Sindbis		Semliki Forest	VSV	
	Expt. 1[d]	Expt. 2		Expt. 1	Expt. 2
0	11,000	6250	955	5150	2500
125	14,500 (130)	5750 (92)	1050 (110)	4160 (81)	1450 (58)
250	7600 (69)	3000 (48)	340 (36)	1250 (24)	1250 (50)
500	4800 (43)	3000 (48)	316 (33)	1100 (21)	1200 (48)
1000	2600 (23)	1900 (30)	165 (17)	830 (16)	455 (18)

	Vaccinia		EMC-2
	Expt. 1	Expt. 2	
0	145	125	7250
125	200 (137)	125 (100)	9550 (132)
250	145 (100)	160 (128)	9100 (125)
500	140 (96)	80 (64)	5750 (79)
1000	80 (55)	105 (84)	6900 (95)

[a] Total-body exposure.

[b] Interferon titer of serum pool, expressed as units per 1.6 ml (6 animals/pool).

[c] Percentage of interferon titer of unirradiated animals given in parentheses.

[d] These results have been published previously (5). We are indebted to J. and A. Churchill Ltd. London, for allowing us to reproduce these figures.

tested as interferon inducers. Induction of circulating interferon was therefore examined with seven more viruses, 4 days after total-body exposure of C_3H mice to 125, 250, 500, and 1000 R. The results of these experiments are summarized in Table II. It is evident that, of all viruses tested, only the 3 myxoviruses gave results similar to those obtained with NDV as inducer. In the case of these 4 myxoviruses, the circulating interferon producing system appears to be quite radiosensitive, a dose of 125 R being sufficient to abolish most of the interferon production. On the other hand, the circulating interferon response to the nonmyxoviruses shows various radiosensitivities. Interferon response to EMC and vaccinia is highly radioresistant, the response to VSV and the two arboviruses is more radiosensitive, but much less than the response to myxoviruses.

Effect of irradiation on circulating leucocytes. The effect of the different doses of irradiation on the number of circulating granulocytes and lymphocytes is summarized in Fig. 1.

Effect of irradiation on interferon induction with NDV in macrophage cultures. Macrophages are able to produce interferon *in vitro* (9, 10), and it has been suggested that they could play a role in interferon production *in vivo* (11). We therefore decided to examine the effect of X-irradiation on *in vitro* interferon synthesis by peritoneal macrophages. Sixteen macrophage cultures were di-

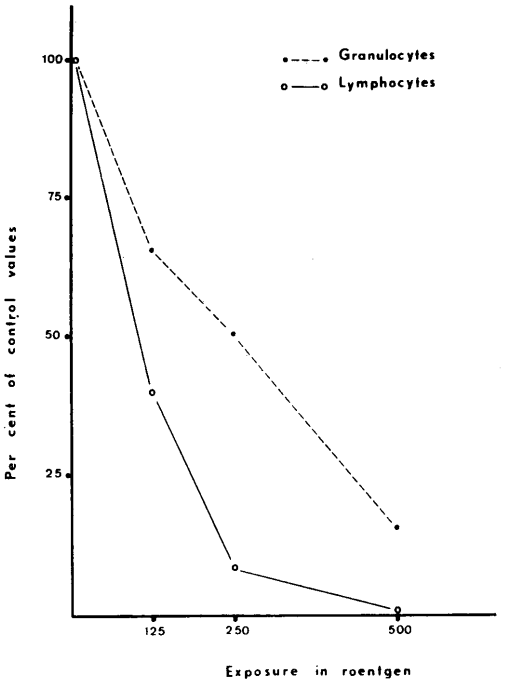

FIG. 1. Influence of the exposure to different amounts of X-rays on the number of peripheral granulocytes and lymphocytes in C_3H mice. Values are given as percentage of control values obtained from unirradiated mice. Each point represents the average of five determinations in different mice. Cells were counted 4 days after irradiation.

vided into 4 groups of 4 cultures each. One group served as control, and the three others were irradiated at a dose of 125, 250, and 1000 R, respectively. The cultures were then incubated at 37° during 4 days. On the fourth day after irradiation, the cells were infected with NDV to stimulate interferon production. The interval of 4 days was chosen because it corresponded to that of the *in vivo* studies. At this time, microscopic examination did not reveal any gross effect of the X-irradiation. The culture medium was aspirated, and 1 ml of an NDV suspension was added to each culture. The input multiplicity was 100 egg ID_{50}/macrophage. The virus inoculum was removed 30 min later and 5 ml of fresh medium was added. Twenty-four hr later a marked cytotoxic effect was present in the virus inoculated cultures, and the culture media were harvested and processed for interferon titrations. The results of this experiment are given in Table III. It is evident that, even at 1000 R, no significant decrease of interferon synthesis has occurred.

TABLE III. NDV Interferon Levels in Irradiated Macrophage Cultures.

Dose of irradiation (R)	Interferon titer of culture fluid
0	132[a]
125	166
250	126
1000	107

[a] Units per 1.6 ml; each value represents the titer of the pooled fluids from four cultures.

Determination of molecular weight of NDV and Sindbis induced interferons. In view of the results, summarized in Table II which demonstrate the existence of circulating interferon producing systems of different radiosensitivities, we decided to compare molecular weights of NDV and Sindbis induced circulating interferons.

This was done by gel filtration on Sephadex G-100 (12). Two ml of pooled mouse sera, obtained from animals 6–8 hr after injection of either Sindbis or NDV, were applied to the top of the column. With either virus as inducer, there was a minor peak of interferon activity corresponding to a molecular weight of 68,000 and major peak indicating a molecular weight of 34,000. These values are within the range of those recently described by Hallum *et al.* (13) for NDV. For Sindbis virus, no previous results are available for comparison.

Discussion. In our first experiments on the induction of circulating interferon in X-irradiated mice (3), NDV and Sindbis virus were chosen as inducers of interferon synthesis because both viruses had been shown by other investigators to be good interferon inducers (1). The differential effect of X-irradiation on interferon induction by the two viruses, as suggested by these experiments, was confirmed in the present study. However, rather than following the effect of a single dose of 1000 R during successive days, we found that a more reliable way of emphasizing the differences in the interferonogenicity of the two viruses was to vary the dose of radiation. When

this procedure was applied to other interferon inducing viruses, the radiosensitivity of circulating interferon production varied widely for different viruses. Thus, the myxoviruses induce a circulating interferon producing system which is very radiosensitive; vaccinia and EMC virus induce a circulating interferon-producing system which is very radioresistant. An intermediate position is taken up by VSV, and by the two arboviruses, Sindbis and Semliki Forest. In this respect, it is of interest that Glasgow found that circulating interferon induction by NDV was much more radiosensitive than was induction by EMC, Sindbis, Chikungunya, or herpes virus (14).

Theoretically, the effect of X-rays on interferon production could be caused either by a direct effect on the interferon synthetizing apparatus at the cellular level, or by a decrease of the number of cells that produce circulating interferon, or by a combination of both mechanisms. The first possibility, a direct effect of the X-rays on interferon production, would seem unlikely, at least at 125 and 250 R, in view of our *in vitro* experiments, where irradiated peritoneal macrophages, exposed to NDV, have shown no inhibition of interferon synthesis at doses of radiation that inhibit 80% or more of NDV induced interferon synthesis *in vivo*. Of course, this argument is not absolute, since *in vivo* and *in vitro* conditions are not the same, and different cell types might be affected differently by the irradiation. A much stronger argument in favor of the second possibility is provided by our previously published experiments in lethally irradiated mice, restored with syngeneic or xenogeneic bone marrow cells, which have shown a good correlation between restoration of hematopoietic function and restoration of circulating interferon synthesis (4). The most reasonable explanation for the phenomenon described in this paper is therefore to ascribe the diminished interferon production, after exposure to 125 or 250 R, to a reduction in number of the interferon-producing cells. Thus it appears that after intravenous injection of a myxovirus suspension, circulating interferon production is mainly carried out by very radiosensitive, bone marrow derived, cells. Both lymphocytes and granulocytes fall into this category [see recent review by Bond *et al.* (15) and by Mathé and Bernard (16)]. Indeed, the number of granulocytes decreases markedly during the days following exposure to 250 R due to mitotic death of the majority of stem cells (15, 17). Moreover, the peripheral lymphocyte is characterized by a unique and very pronounced susceptibility to a direct cell killing effect of the X-rays (interphase death) (18). Our own counts of circulating lymphocytes and granulocytes in C_3H mice, as shown in Fig. 1, demonstrate this effect of the X-rays on circulating leukocytes. The highly inhibitory effect of a dose of X-rays as low as 125 R, on serum interferon production with myxoviruses, suggests that lymphocytes, rather than granulocytes, are mainly involved. In this regard some recent experiments by Wheelock are particularly relevant; he found that *in vitro* the lymphocyte fraction of human peripheral blood produced interferon in response to NDV, whereas polymorphonuclear leukocytes were unable to do so (19). Similar results were obtained by Cantell and coworkers, using Sendai virus as inducer (20). We hope to be able to obtain more direct *in vivo* information about this point by carrying out interferon induction studies in irradiated animals which will subsequently have received lymphocyte suspensions.

Since our experiments have shown the existence of circulating interferon producing compartments of different radiosensitivities, it is surprising that in the irradiated animals, once the radiosensitive compartment was eliminated, the myxoviruses did not induce interferon production in the more radioresistant compartment, especially since our *in vitro* experiments have shown that irradiated macrophages, which belong to this radioresistant compartment (21), are able to produce interferon upon stimulation by NDV. We have no ready explanation for this paradox. Maybe the property of myxoviruses of being readily adsorbed to erythrocytes (22, 23) is responsible for preventing the myxoviruses of being massively taken up by the more radi-

oresistant interferon producing compartment. This property could also explain the large contribution of radiosensitive, bone marrow derived, cells to serum interferon production with these viruses, since there is evidence that leukocytes and erythrocytes share the same receptors for myxoviruses [see recent review by Gresser and Lang, (24)]. Studies are under way to clarify this problem. As to the nonmyxoviruses examined in this study, our results only allow to conclude that they induce more radioresistant circulating interferon producing systems than do myxoviruses, without giving any definite clue as to which cells are involved. The molecular weights of the Sindbis-induced serum interferons, comparable to those of the NDV-induced, indicate that the difference in radiosensitivity of the producing system does not necessarily entail the production of different molecular species.

Summary. The effect of X-irradiation on circulating interferon induction was examined in C_3H/He mice, 4 days after total-body exposure to 125, 250, 500, or 1000 R. Nine viruses were tested as inducer: Newcastle disease, influenza A, Sendai, mumps, Sindbis, Semliki Forest, vesicular stomatitis, encephalomyocarditis, and vaccinia. Interferon induction by the four myxoviruses was very radiosensitive, exposure to 125 R being sufficient to reduce serum interferon levels to values ranging between 4 and 28% of levels in control animals. In contrast, interferon induction by either encephalomyocarditis or vaccinia viruses was very radioresistant, and even after 1000 R significant amounts of circulating interferon were produced. Circulating interferon induction by vesicular stomatitis, Sindbis and Semliki Forest was of intermediate radiosensitivity. The high radiosensitivty of myxovirus-induced circulating interferon production suggests an involvement of lymphocytes in circulating interferon induction by these viruses.

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