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Sparing Effect of X-Rays for Mice Inoculated Intranasally with Egg-Adapted Influenza Virus, CAM Strain.* (29720)

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The final amount of pneumonia in mice inoculated with influenza virus depends on the strain of mice(1), degree of adaptation of the virus(2,3,4), and amount of virus in the inoculum(5,6). Viruses that are adapted to mice cause pneumonia after serial passage from mouse to mouse. Small doses of adapted viruses multiply to high titer in lungs and cause deaths, while equal amounts of unadapted virus may have little evident effect. Nevertheless, large doses of active unadapted virus can cause lesions in the lungs(6,7,8), by a direct toxic effect on cells apparently not dependent on multiplication of viruses within the cells.

The effect of ionizing radiation on 2 of these variables has been examined in other studies, but the total information is meager. Exposure of several stocks of albino and black agouti mice to X-rays before infection increased their susceptibility to type B influenza virus unequally(1). The radiation had the greater effect on the black agouti stocks which normally were more susceptible to the virus than the albino strains. In addition, X-rays and γ-rays increased virus proliferation in mice inoculated with type A and type A-prime strains(9,10). However, no observations on susceptibility to viral toxicity have been described.

The objective of the present study was to ascertain the effect of prior exposure to X-rays on the course of the pneumonia caused by the toxic effect of influenza virus in mice. Therefore, the virus employed was an egg-adapted line of CAM virus, an A-prime strain which causes pneumonia and even death although it is not adapted to mice(8). The findings indicate that pretreatment with a large sub-lethal dose of X-rays had a sparing effect on production of fatal pneumonia.

Materials and methods. A. Mice. Mice used in these experiments were CF-1 adult albino males weighing approximately 25 g. Irradiated and non-irradiated mice were inoculated intranasally with infected allantoic fluid containing egg-passaged CAM A-prime strain of influenza virus. The pneumotoxic effect of the virus was ascertained in terms of the risk of developing lung lesions and the risk of dying. All experiments were performed during summer months.

Animals dying before the end of an experiment were autopsied as soon as they were discovered in order to establish the cause of death. The occurrence of influenzal lung lesions at autopsy was accepted as presumptive evidence of death due to toxic pneumonitis regardless of the extent of pneumonia.

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On the other hand, deaths occurring without associated lung lesions were designated deaths from X-ray. Survivors to 17 days were autopsied and presence or absence of lung lesions noted.

B. X-Irradiation. Mice were exposed to 350R whole-body X-irradiation one day before inoculation of virus. X-rays were produced by a General Electric Maxitron machine operated at 250 kV using 0.25 mm copper and 1.0 mm aluminum added filtration to obtain a beam with a half value layer of 0.8 mm copper. The mice were exposed at a rate of approximately 33R per minute as measured in air. During X-irradiation the mice were held in individual plastic capsules perforated to permit free circulation of air. Non-irradiated mice were handled in the same manner as the irradiated mice but were sham-irradiated with the X-ray beam turned off.

C. Inoculation. One day after X-irradiation, the mice were lightly anesthetized with ether and were inoculated intranasally with 0.08 cc volumes of undiluted allantoic fluid containing freshly harvested virus and 1000 units of penicillin and 1 mg streptomycin per cc. Other mice employed as controls for possible effects of X-rays alone were inoculated with sterile beef broth also containing penicillin and streptomycin.

D. Virus content of lungs. The virus content of infected lung tissue was ascertained by titration in eggs. Ten percent suspensions of pooled lung tissue were stored at −60°C until completion of an experiment. The frozen materials were thawed, diluted in sterile broth containing 1000 units of penicillin and 1 mg of streptomycin per cc, and inoculated intra-allantoically into 10-day-old embryonate eggs. Seventy-two hours after inoculation, allantoic fluids from individual eggs were tested for the presence of hemagglutinating virus. The 50% egg infectivity (EID_{50}) was computed by the method of Reed and Muench(11).

E. Hemagglutination-inhibition tests. Hemagglutination-inhibiting (HI) activity of pooled sera was measured by standard methods(12).

Results. I. Morbidity and mortality. Irradiated and non-irradiated mice responded to intranasal inoculation of egg-passaged CAM influenza virus with development of plum-colored hilar lesions involving all lobes of the lungs. As reported by Sugg(8), the gross appearance of toxic pneumonitis was indistinguishable from that caused by mouse-adapted lines of influenza virus. Characteristically, almost all mice killed by the toxic pneumonitis exhibited consolidation of the entire lung except a few irradiated mice in which the process had not progressed to completion by the time of death. On the other hand, 12 irradiated mice died without evident pulmonary pathology even though inoculated with virus. This number represented about 5% of inoculated, irradiated mice and was substantially the same percentage mortality as that observed in radiation controls given broth. Therefore, the 12 were classed as deaths due to X-ray alone and were omitted from computations of morbidity or mortality due to pneumonia.

TABLE I. Sparing Effect of X-Irradiation on Toxic Pneumonitis Caused by Unadapted Influenza in Mice.

	Morbidity*		Mortality†	
Exp	Irradiated	Non-irradiated	Irradiated	Non-irradiated
1	11/20	20/20	9/20	18/20
2	20/20	20/20	17/20	20/20
3	19/20	15/16	16/20	14/16
4 a	14/20	18/19	13/20	17/19
b	14/16		11/16	
5	6/17	20/20	5/17	12/20
6	16/20	15/20	14/20	15/20
7‡	6/19	14/19	5/19	12/19
8	13/16	20/20	11/16	20/20
9§	19/20	17/20	18/20	16/20
Total	138/188	159/174	119/188	144/174

* No. with pneumonitis/No. at risk.
† No. of influenzal deaths/No. at risk.
‡ 3/20 irradiation controls killed by x-ray alone.
§ 5/16 irradiation controls killed by x-ray alone.

Intranasal inoculation of virus caused lung lesions in 91% (159/174) of non-irradiated mice but only in 72% (138/188) of irradiated mice (Table I). The overall reduction in morbidity was 19% ($\chi^2 = 19.2$, $p<0.002$), individual experiments exhibiting greater or lesser X-ray effects according to the relative radiosensitivity among groups of mice in different tests. In total, 83% of non-irradiated mice (144/174) died but pretreatment with

TABLE II. X-ray Induced Delay of Deaths Among Mice Inoculated Intranasally with Egg-Adapted CAM Strain of Influenza Virus.

Day of infection	No. of deaths/day: Non-irradiated observed	Irradiated Observed	Irradiated Expected*	Significance χ^2	Significance p†
2	15	3	11.5	6.3	<.01
3	59	22	49.0	14.9	<.001
4	36	20	30.0	3.3	<.10
5	14	19	11.5	6.3	<.01
6	10	13	8.3	2.7	<.10
7	3	11	2.5	29.0	<.001
8	3	12	2.5	36.0	<.001
9	1	7	.7	49.0	<.001
10 or more	3	12	2.5	36.0	<.001
Total	144	119	119		

* No. expected = % mortality in non-irradiated mice times total No. of irradiated mice.

† Probability of observed No. of deaths occurring by chance.

X-rays reduced the mortality to 63%, 119/188 ($\chi^2 = 17.2$, $p<0.001$). The sparing effect on mortality was observed to some extent in all experiments but one. However, in that experiment (No. 9, Table I) there was an associated higher risk of death from X-ray than that customarily observed with 350R. Taken together the data indicate that pretreatment with X-rays reduced the risk of developing pneumonia and favored survival.

The ratio of morbidity to mortality, *i.e.*, the case fatality rate, for non-irradiated mice was 91% (144/159) and that for irradiated mice was 86% (119/138), $\chi^2 = 1.2$, $p>0.10$. The absence of a significant difference was taken as evidence that pretreatment with X-rays did not influence the frequency of death among mice in which the toxic pneumonia developed. However, it was evident that pretreatment X-rays reduced the frequency with which pneumonia developed, and as a result also reduced mortality since the case fatality rate was unchanged. Most of the deaths due to the toxic effect of the virus occurred from 2 to 8 days after inoculation (Table II). The observed daily number of deaths in the irradiated mice was compared with the number expected in a non-irradiated population of equal size to ascertain effects of X-rays on the course of the disease. The principal results were a reduction in number of deaths on days 2 and 3 and an increase in proportion from day 7 and on. Evidently, pretreatment with X-rays retarded pathogenic processes concerned in development of fatal pneumonitis.

II. Virus content of lungs. In general, lethal effects of allantoic fluids containing egg-adapted CAM virus correlate with amounts of live virus introduced into the lungs of recipients at time of inoculation(6). The finding that mortality rates in irradiated mice were lower than those in the non-irradiated controls suggested the possibility that pretreatment with X-rays reduced the amount of intranasally administered virus actually entering the lower respiratory tract. Therefore in several experiments additional mice were employed for the specific purpose of obtaining data concerning the virus content of the lungs immediately after inoculation and at various intervals thereafter. Irradiated and non-irradiated mice were sacrificed at 0, 1, 2, 4, 6, 11, and 16 days after inoculation. Lungs from 3 mice per group were removed aseptically, pooled, ground with alundum and prepared as a 10% suspension in penicillin and streptomycin broth. The lung suspensions were stored in a freezer until withdrawn for in ovo titrations.

Following intranasal inoculation of a highly adapted virus, for example, influenza type A, PR8 strain, there is a rapid increase of virus in lungs reaching peak titers in 24 to 48 hours(5). In contrast, in the present study maximum titers of unadapted CAM virus in non-irradiated controls were observed immediately after inoculation (Table III). Subsequently, the amount of virus in lungs of the controls progressively declined until by 11 days none was detected.

The findings in the irradiated mice agreed closely with those in the non-irradiated controls, at least for the first week after inoculation. EID_{50} titers of lung suspensions obtained from irradiated mice immediately after inoculation were almost the same as those in the controls indicating that equal amounts of toxic materials were introduced into the lower respiratory passages of both groups. From days 1 to 6 also, EID_{50} titers in irradiated mice were virtually identical to those in non-irradiated controls. On the other hand, titers

TABLE III. Virus Titers in Lungs and HI Responses of Irradiated and Non-Irradiated Mice Inoculated Intranasally with an Unadapted Line of Type A-Prime Strain (CAM) Influenza Virus.

Exp	Mice	EID_{50} titer* of lung at intervals after intranasal inoculation (days)							HI response	
		0	1	2	4	6	11	16	(day)	Titer
I	Irradiated	6.5	6.4	n.d.	4.2	n.d.	4.0	n.d.	(11d)	1:32
	Non-irradiated	6.0	6.8	n.d.	4.3	n.d.	<2.0	n.d.	(11d)	1:1024
II	Irradiated	7.4	7.9	n.d.	4.9	n.d.	3.4	n.d.	(11d)	1:32
	Non-irradiated	7.1	7.3	n.d.	6.5	n.d.	<2.0	n.d.	(11d)	1:256
III	Irradiated	n.d.	8.9	7.4	n.d.	6.2	n.d.	<2.0	(13d)	1:8
	Non-irradiated	n.d.	8.7	7.4	n.d.	6.4	n.d.	<2.0	(13d)	1:32

* $Log_{10}x$.

of $10^{-3.4}$ and $10^{-4.0}$ at 11 days indicated persistence of virus in the lungs of irradiated mice. By 16 days, clearance of virus from the lungs of both groups was almost complete. Clearly, the overall reduction of mortality due to pneumonitis during the acute phase of the disease was not accompanied by a corresponding reduction in the level of virus in the lungs of X-irradiated mice; if anything, virus was detected over a longer interval in the lungs of irradiated mice than in the lungs of the controls.

III. Hemagglutination-inhibiting response. Pretreatment with X-rays has been shown both to delay and to reduce HI responses of mice to infectious virus and inactivated influenza virus vaccines(9,10,13,14). In the present study, pretreatment with X-rays also reduced HI titers of pooled sera from groups of 3 mice sacrificed 11 or 13 days after inoculation (Table III). The serum pools obtained on the 11th day were from the same mice furnishing lung tissue for virus titrations.

Discussion. These findings demonstrate a favorable influence of X-rays on the outcome of toxic pneumonia in mice inoculated with egg-passaged CAM influenza virus. There are also other lines of evidence that exposure to ionizing radiation may have beneficial effects on the outcome of virus infections. For example, exposure to X-rays before initiation of infection reduced the frequency of death among mice inoculated with St. Louis encephalitis virus(15) or with lymphocytic choriomeningitis virus(16,17). Pretreatment with X-rays also favored survival for 10 days of mice inoculated with swine influenza virus (18). Unfortunately, the effect on final mortality was not learned in that experiment because observations were terminated before the disease in irradiated mice had run its full course.

Although the primary effect of the radiation was to prevent lung lesions, it had no evident effect on virus titers in the initial stages of the disease process. The absence of an X-ray effect on virus titers during the first week suggests that pretreatment with X-ray did not impair innate processes involved in clearance of virus from the lungs. Furthermore, the observation that equal amounts of virus were introduced into lungs of irradiated and non-irradiated mice indicated that reduced morbidity was due either to increased resistance of irradiated lung tissue to the toxic action of the virus or to reduced capacity of irradiated mice to respond to a given tissue injury. Although there is little precedent for the first possibility, one example is the increased survival time observed in mice injected with a lethal dose of botulinum toxin 24 hours after whole body exposure to 760R(20).

On the other hand there are several means by which X-ray might reduce the response to injury. X-ray is known to exert anti-inflammatory activity: an effect which has been employed with some success for treatment of well-localized bacterial infections(21) and has also been described as a means for promoting regression of ocular lesions induced by vaccinia virus in rabbits(22). An anti-inflammatory effect is also suggested by the distinct parallelism between the results of pretreatment with X-rays in the present

study and the reported effects of xerosin, a microbial product on CAM pneumonitis in mice(23). Xerosin nonspecifically suppresses inflammation by preventing edema, hemorrhage and cellular infiltration(24), and also prevents lung lesions in mice inoculated with influenza virus without evident effect on virus multiplication. Xerosin exerts its effect principally during the first week of the experimental disease temporarily decreasing mortality as well as morbidity and it was precisely during that early period that pretreatment with X-ray reduced deaths due to the toxic pneumonia most dramatically.

X-ray is also known to suppress immunologic phenomena. Depression of immune processes in the irradiated mice may have enhanced the anti-inflammatory effect by interfering with specific cellular responses in a manner analogous to that postulated as an explanation for the protective effect of X-rays for mice inoculated with lymphocytic choriomeningitis virus. However, there is no clear evidence that acquired immunity is causally related to the pathogenesis of influenzal pneumonia during the first week of infection; if anything, active and passive immunization are generally considered to be protective.

X-ray, in the dose employed in this study, is also known to depress blood levels of properdin thereby increasing susceptibility of mice to bacterial infections(25). The sparing effect observed in this study suggests that properdin may not be an important factor in *in vivo* resistance to influenza virus even though it is known to potentiate virus neutralization by normal serum.

Our results are at variance with other investigations reporting the absence of an effect of X-rays on experimental influenza(14,19) and with those that report an increased susceptibility(1,9,10). The differences in results might be due, in part, to the use of different stocks of mice or to differences in degrees of adaptation of various strains of viruses employed. Moreover, the sparing effect on infection with highly adapted strains of virus is less likely to be demonstrable because the radiation concomitantly increases virus proliferation.

Summary. Pretreatment with X-rays by whole-body exposure to 350R delayed the time of death and reduced the number of deaths in mice resulting from intranasal inoculation of large doses of egg-passaged CAM strain influenza virus. The decreased mortality seemed to be due to a reduction in the frequency with which toxic pneumonitis developed after inoculation of virus since the radiation did not change the case fatality rate. The most likely explanation for the sparing effect is that pretreatment with X-rays prevented a severe inflammatory response to the injury caused by a toxic principle associated with the virus.

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Vulnerability of the Subterminal Chromosomes of Cultured Rat Mammary Cancer Cells to Alterations.* (29721)

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In addition to a pair of sex chromosomes the rat has 20 pairs of autosomes. Unlike the mouse, which has all terminal chromosomes, the rat has 5 *subterminal*, 9 *terminal* (including the sex chromosomes), and 7 *median* pairs of chromosomes. This morphologic division facilitates cytologic study.

Cells from mammary cancers induced in young female Holtzman rats by polynuclear hydrocarbons have been grown in tissue culture. Permanent cell lines from 2 cancers were obtained and chromosome studies of the cultured cells performed. Although many of the cells maintained a diploid number, deviations from a normal karyotype were found frequently. Most of the alterations which occurred were in the group of subterminal chromosomes.

Methods. One of the cell lines (designated #21) originated from a cancer induced by the gastric instillation of 3-Methylcholanthrene(1) and has been in culture since August 1961. The other line (#31) was derived from a cancer induced by feeding 7,12 Dimethylbenz(a)anthracene(2) and this line has been in culture since February 1962. For primary culture, cells were dispersed from the cancers with 0.2% trypsin (Difco 1/250)-0.08 M versene solution. The culture medium was 20% serum (calf serum for the #21 line, horse serum for the #31 line) and 80% Eagle's minimum essential medium containing a doubled concentration of amino acids and vitamins in addition to streptomycin (50 μg/ml) and penicillin (30 units/ml). No other anti-microbial agents were used. The method of Chanock *et al*(3) was used to culture for PPLO (pleuropneumonia-like organisms) in the medium of both cell lines and to date these microorganisms have not been detected.

At the time of chromosome studies, cells of the #21 line had been cultured for 10-15 months with 15-36 transfers and cells of the #31 line had been in culture 7-10 months with 10-23 transfers. The cells were grown on the 30 cm^2 flat bottom Bellco tissue culture tubes containing 3 ml of culture medium. Cells were transferred by detaching and dispersing with 0.2% trypsin, centrifuging, resuspending in fresh culture medium, and inoculating new flasks with 1-2 $\times$ 10^5 cells for each ml of medium in the flask. Later, when the cells were established, the inoculum was reduced to 1-2 $\times$ 10^4 cells per ml medium. For chromosome analyses cells were inoculated into plastic Petri dishes (35 mm diameter) containing a 22 mm square cover slip on the bottom. Near the end of the logarithmic growth phase, chromosome spreads were prepared by the hypotonic procedure of Tjio, Puck and Robinson(4). To arrest mitosis, colchicine in an amount to provide 0.5 μg/ml (1.3 $\times$ 10^{-6} M) was added 4 hours prior to the hypotonic treatment. Cells were stained by the aceto-orcein technique of LaCour(5) with the exception that 2% synthetic orcein in 70% propionic acid was used. The slides were carefully examined and chromosome

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