The Influence of X-Irradiation on Survival and Interferon Levels in Viral Infected Mice.* (33036)

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Reports describing an enhanced susceptibility and mortality in viral infection of mice pretreated with X-irradiation (1,2) have appeared in previous literature. This greater susceptibility has been attributed to the suppression of the reticuloendothelial system (RES) and the immune response. It has further been suggested that leukocytes and /or circulating macrophages might contribute to the host defense mechanism during certain phases of viral infection by producing and/or distributing interferon or other viral inhibitory substances to sites of viral replication (3).

By employing X-irradiation, splenectomy, ethyl stearate (4), or iodoacetate (5) therapy, alone and in combination, this study was undertaken to determine the contribution of the RES to overall host defense in influenza infected mice. The influence that this depression of the RES had on interferon production and host survival is also described.

Materials and Methods. The C57Bl6J specific pathogen-free mice utilized for these studies were bred in our laboratories. They are routinely tested for ecto- and endoparasites, the presence of Pseudomonas and Salmonella, and for eight of the most prevalent murine viruses (6). Even after 3 years of breeding and testing, these animals have been kept free of any of these infectious agents.

The mice were intranasally infected with $10^{1.5}$ and $10^{5.5}$ LD_{50} doses of PR8 type A influenza virus. A certain portion of these animals were X-irradiated 3 days previously with 425 R at a dose rate of 160 R/min, [LD_{50} is 654 R for males and 647 R for females (7)], utilizing a General Electric 300-kV X-ray apparatus. The animals were irradiated in a compartmentalized Lucite turn table. During initial experiments mice were orbitally bled at 24-hour intervals, 3 mice/sex, per point for total leukocyte determination, utilizing a Fisher Autocytometer. No animal was ever bled more than once, in order to assure that WBC counts were not influenced by a depletion of, or alteration in cell counts due to this mechanical manipulation.

Infectivity titers of the PR8 virus were determined by intranasal inoculation and subsequent Reed-Muench calculation of the LD_{50}.

Upon completion of the LD_{50} determination in irradiated, nonirradiated, splenectomized irradiated and nonirradiated, and ethyl-stearate-treated animals, a series of experiments was inititated to follow comparative interferon levels in lung tissue and sera of irradiated and nonirradiated mice infected with PR8 virus. Seventy-two hours elapsed after irradiation of the animals, prior to their exposure to $10^{1.5}$ and $10^{5.5}$ LD_{50} doses of influenza virus. Five hours after intranasal infection, a group of 5 male and 5 female animals were exsanguinated. The lungs were harvested at 4°C and pooled according to sex from these groups of animals, as were the sera. At subsequent 24-hour intervals through 120 hours, the same procedure was followed. The lung tissue and sera were stored frozen at —20°C and at the end of this portion of the experiment, 10% suspensions of lung tissue in Hanks' BSS containing 50 μg of streptomycin and 50 units of penicillin were prepared and centrifuged at 10,000*g* for 10 min. In another series of experiments a similar group of animals, irradiated and nonirradiated, were intravenously inoculated at 24-hour intervals for 72 hours prior to viral

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infection with ethyl stearate, according to a procedure reported by Stuart *et al.* (4), in order to suppress the reticuloendothelial phagocytic function. Circulating macrophage phagocytic activity was also suppressed by a modified procedure utilizing iodoacetate (5). Animals were intravenously inoculated with 1.2 μM iodoacetate/animal at 24-hour intervals for 72 hours prior to viral infection. The 72-hour inoculation was followed 1 hour later by intranasal infection with virus. One hour after that and at 24-hour intervals, the mice again were inoculated with 1.2 μM iodoacetate.

Phagocytic activity of mouse macrophages was determined following the above regime of ethyl stearate or iodoacetate administration. Forty-eight hours and 24 hours prior to phagocytic index determination, mice were intraperitoneally inoculated with 1% gelatine to increase the histiomonocytic cells. Prior to harvesting the peritoneal exudate cells, 1 ml of opsonized sheep erythrocytes were inoculated intraperitioneally, and 1 hour later 5 ml of buffered saline were inoculated intraperitoneally. The peritoneal washings were then harvested, and centrifuged, and smears were made of the exudate. The phagocytic index was determined by counting 100 macrophages.

The contribution of the spleen to recovery from PR8 virus infection and interferon production was also determined. Mice were splenectomized and allowed to recover for 7 days, at which time a group of these animals were irradiated and infected as described above. Five- and 24-hour lung and sera interferon levels were then determined.

The interferon titrations were carried out by using L-M cell monolayers and a Mengo virus assay system. The L-M cells were initially propagated in suspension culture in 199-0.4% peptone media in the absence of antibiotics (8). Plaque bottles were prepared from these suspension cultures by seeding tablet bottles as previously described (9) with 4 ml of 8 $\times$ 10^5 L-M cells/ml. The cells were allowed to attach for 36 hours, the growth media were decanted, and 1 ml of twofold dilutions of influenza-infected mouse lung tissue or mouse sera was added to at

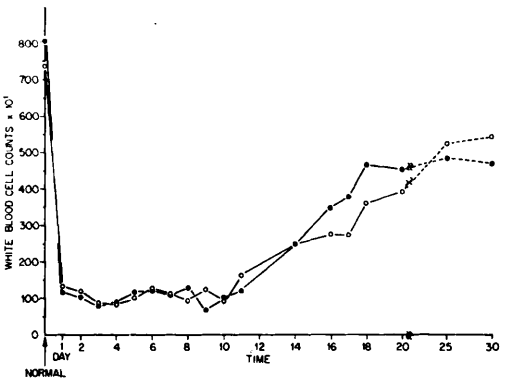

FIG. 1 White blood cell counts of X-irradiated mice. (○), male animals; (●), female.

least three plaque bottles per dilution. These bottles were then incubated for 18 hours at 34°C, the monolayers were washed twice with Hanks' BSS, and a dilution of Mengo virus was added to give 30 to 50 plaques/control bottle. The virus absorbed for 1 hour at 34°C, and was then overlayed with 3 ml of nutrient agar containing 1 $\times$ Eagle's BME, 0.1% horse serum, 1.12% protamine sulfate, and 1.0% agar, suspended in indicatorless Hanks' BSS. After 48 hours of incubation, a neutral red overlay was added and the plaques developed further for another 12 hours at 34°C. The 50% plaque reduction assay (10) was utilized to determine the levels of interferon in lung tissue.

To confirm that the antiviral material in the mouse lung tissue belonged to the class of interferons, we used the following criteria: (i) Dialysis for 24 hours against a pH 2.0 HCl-saline buffer and back dialysis to pH 7.4; (ii) centrifugation at 100,000*g* for 2 hours; (iii) lack of protection afforded by the substance to HeLa cells infected with poliovirus; and (iv) destruction of interferon properties by treatment with trypsin.

Results. The depression of circulating leukocytes after 465 R X-irradiation in male and female mice is shown in Fig. 1. As previously reported (11), this is a very rapid 7- to 8-fold decrease, primarily of lymphocytes, during the first 24 hours after irradiation. In both sexes the WBC remains depressed at this level for 10–11 days and then gradually rises over the next 20 days. In no case did we observe any deaths during the 30 days of

TABLE 1. The LD_{50} Response of Male and Female Mice When Challenged by the Intranasal Route with PR8 Influenza Virus

Treatment	LD_{50} dose	
	Male	Female
Irradiated	6.3	6.9
Nonirradiated	6.2	6.5
Irradiated and ethyl stearate	6.4	6.7
Nonirradiated and ethyl stearate	6.3	6.6
Irradiated and splenectomy	6.4	6.5
Nonirradiated and splenectomy	6.2	6.6

observation of these mice. The LD_{50} response of mice to intranasal infection with PR8 influenza virus in irradiated and nonirradiated chemically treated and splenectomized animals is seen in Table I. We note no significant difference in the LD_{50} response of mice irradiated or nonirradiated. There was a slight difference in the LD_{50} dose of male and female animals, with female being more susceptible. We also noted this in the LD_{50} determination of the nonirradiated animals, and during aerosol infection experiments (in a later report) of 4000 male and female animals infected with this virus. Depression of phagocytic function by the inoculation of ethyl stearate, which proved 40–50% effective, had no effect on the LD_{50} of mice as is also shown in Fig. 1. Splenectomy similarly did not influence recovery.

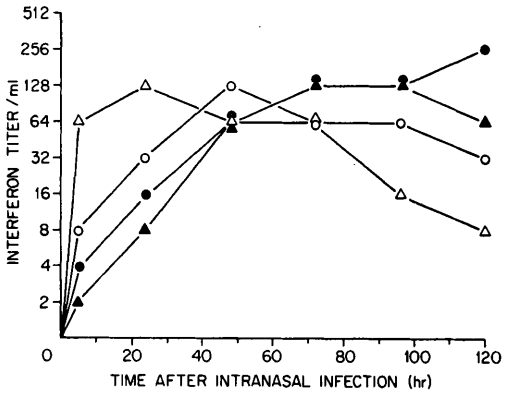

FIG. 2. Interferon titers in male lung tissue of nonirradiated and irradiated mice. (△), nonirradiated mice and virus dose $10^{5.6}$; (▲), nonirradiated, virus dose $10^{1.5}$; (○), irradiated, virus dose $10^{5.6}$; (●), irradiated virus dose $10^{1.5}$.

The influence that irradiation has on levels of interferon in lung tissue of C57Bl6J male mice when compared with nonirradiated animals is demonstrated in Fig. 2, and for female mice in Fig. 3. Although no appreciable differences can be detected in levels of interferon produced by either sex, a trend in the rate of interferon production is suggested when comparing irradiated animals. This difference, though relatively small, is more discernible during the first 24 hours after infection in those animals receiving a higher dose of virus. No interferon levels were detected in sera. Further impairment of the RES by the use of ethyl stearate or iodoacetate, or surgical impairment did not influence the interferon response.

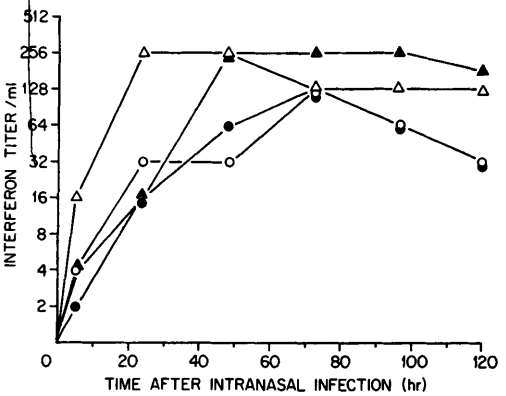

FIG. 3. Interferon titers in female lung tissue of nonirradiated and irradiated mice. Symbols same as in Fig. 2.

Discussion. Our intent was to evaluate the contribution of the lymphocytes and circulating macrophages on host defense and interferon production when mice were infected by a presumably normal route. It is clear that if radiosensitive leukocytes are contributors to the host defense mechanism during viral infection, this contribution is not a major factor in the overall host defense as expressed by an LD_{50} response. Furthermore, if phagocytic macrophages are involved in the host response to viral infection, a significant number of them can be nonfunctional before this adversely affects the host mortality. Obviously, since a complete suppression of phagocytes was not achieved by ethyl stearate or iodoacetate treatment, their full contribution

cannot be evaluated. The inability of the macrophage to phagocytize may not be an indication of its total functional characteristics. Moreover, removal of the spleen, an organ which significantly contributes to the number of circulating macrophages, similarly did not influence host mortality.

In a recent report it was suggested that X-irradiation appreciably influences the interferon response of intravenously infected animals (12). Our results can neither fully confirm nor refute these observations, since the Jullien study subjected mice to a lethal dose of X-irradiation, and presumably evaluates only the circulating levels of interferon. Whether other tissues are, after 5 hours of stimulation, synthesizing interferon in the aforementioned studies is difficult to determine, although there are reports of better interferon-producing tissue than the circulating leukocytes (13). Due to the relatively low titers of interferon detected in the lung tissue of the C57Bl6J mouse infected with the PR8 influenza virus, and the rather narrow range of difference between irradiated and nonirradiated mice, our results can only be suggestive. Since the tissue initially challenged with virus contributes interferon, interferon may be providing the host with adequate quantities of these substances for it to successfully respond to an infection challenge. Our studies certainly indicate that radiosensitive host defense tissues are not required during acute viral influenza infection in order for the host to successfully react to such an infection.

Summary. We investigated the contributions of circulating macrophages and lymphocytes to survival of a host exposed to high doses of PR8 influenza virus. It was determined that when using host survival as an index, suppression of these cells and/or their functions had little influence on survival. Interferon levels when measured in sera and lung tissue of mice, showed no significant alteration in irradiated animals. The sex of the animal and splenectomy similarly did not influence these results.

1. Cheever, F. S., J. Immunol. **71**, 431 (1953).
2. DeGara, P. F. and Furth, J., J. Immunol. **50**, 255 (1945).
3. Gresser, I., Proc. Soc. Exptl. Biol. Med. **108**, 799 (1967).
4. Stuart, A. E., Biozzi, G., Stiffel, C., Halpern, B. M., and Mouton, D., Brit. J. Exptl. Pathol. **41**, 599 (1960).
5. Sbarra, A. J. and Karnovsky, M. L., J. Biol. Chem. **234**, 1355 (1959).
6. Parker, J. C., Tennant, R. W., and Ward, T. G., Natl. Cancer Inst. Monograph **20**, 25–36 (1966).
7. Oak Ridge, Natl. Lab. Progr. Rept. 3768, **1965**. 140.
8. Kuchler, R. J. and Merchant, D. J., Univ. Mich. Med. Bull. **24**, 200 (1958).
9. Hellman, A., Kalter, S. S., and Daye, G. T., Acta Virol. **9**, 224 (1965).
10. Wagner, R. R., Bacteriol. Rev. **24**, 151 (1960).
11. Bloom, M. A., Radiology **49**, 344 (1947).
12. Jullien, P. and DeMaeyer, E., Intern. J. Radiation Biol. **11**, 567 (1966).
13. Van Rossum, W. and DeSomer, P., Life Sci. **5**, 105 (1966).

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