NATURAL, GENETICALLY DETERMINED RESISTANCE TOWARD INFLUENZA VIRUS IN HEMOPOIETIC MOUSE CHIMERAS
Role of Mononuclear Phagocytes*

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Mononuclear phagocytes are among the first target cells encountered by invading viruses during infection. It is generally thought that this initial virus-host cell interaction is important for the pathogenesis of viral diseases, and that the capacity of a virus to multiply in phagocytic cells represents a crucial step toward viral attack of parenchymal cells leading to severe disease (1, 2).

In cases of innate resistance of certain inbred mouse strains against defined viral infections, macrophages are found to be resistant in vitro (3–5). Hence, it is tempting to attribute in vivo resistance of such animals to the presence of resistant cells of the mononuclear phagocyte system acting as an in vivo barrier to the spread of infectious viruses.

We have previously described the natural resistance of mice to several orthomyxo-viruses, determined by the dominant gene Mx (6). Myxovirus resistance of A2G mice, homozygous for Mx, develops shortly after birth (7) and is expressed in several organs, as demonstrated by infection with pneumotropic (6), neurotropic (6), or hepatotropic (8) variants of mouse-adapted influenza viruses. This suggests the existence of some systemic factor which matures early in life and provides antiviral protection in vivo. Intact immune responses, including normal T-cell functions (9) and orderly antibody formation (8, 9), are not required for the phenotypic expression of resistance. However, peritoneal exudate macrophages from resistant animals proved to be resistant to in vitro infection with M-TUR,1 a macrophage-adapted influenza-virus-A/Turkey/England/63 strain that readily produces a marked cytopathic effect (CPE) in macrophages from susceptible mice (5). A clear correlation between in vivo resistance of individual mice and in vitro resistance of their macrophages was found in segregation analyses using backcrosses between resistant (A2G × A/J)F1 hybrids and susceptible A/J (5).

These findings were compatible with the idea that macrophages might be instrumental for in vivo resistance in this model system.

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1 Abbreviations used in this paper: CPE, cytopathic effect; HA, hemagglutinin; HKH, influenza virus A/Hong-Kong/1/68; i.e., intracerebral; i.n., intranasal; i.p., intraperitoneal; i.v., intravenous; LD50, mean lethal dose; m.o.i., multiplicity of infection; M-TUR, macrophage adapted influenza virus A/Turkey/England/63; NWS, Stuart-Harris strain of neurotropic influenza virus A; PBS, phosphate-buffered saline; TURH, hepatotropic influenza virus A/Turkey/England/63.
In lethally irradiated animals, macrophages can be replaced by reconstitution with viable bone-marrow cells (10-13). Reciprocal exchange of marrow stem cells between H-2-identical mouse strains, one susceptible to, the other resistant to influenza virus infection, should allow the testing of the importance of macrophages in mediating host resistance. Resistant A2G and susceptible A/J mice have the same set of major histocompatibility loci (14).

The present repopulation experiments demonstrate that, in radiation chimeras, not only peritoneal macrophages, but also Kupffer cells were of bone-marrow origin because both cell types expressed the phenotype of the donor. We further demonstrate that these marrow-derived macrophages were not capable of converting the in vivo resistance phenotype of the host.

Materials and Methods

**Mice.** A/J, CBA/J and (A/J × CBA/J)F₁ mice were obtained from G. L. Bomholtgård, Ry, Denmark. Inbred A2G mice, homozygous for the dominant resistance allele Mx (5), and heterozygous (A2G × A/J)F₁ or (A2G × CBA/J)F₁ hybrids were bred locally.

**Chimeras.** Irradiated bone-marrow chimeras were prepared as previously described (15). Briefly, recipient mice, aged 8-12 wk, were irradiated with 850-950 rad and reconstituted with 3 × 10⁶ viable bone-marrow cells from femurs and tibias of sex-matched histocompatible donors. Chimeras were used 4, 12, 24, or 44 wk after reconstitution as indicated in the text.

**Virus Strains, In Vivo Challenge, and Titrations.** M-TUR was a macrophage-adapted strain of A/Turkey/England/63 (Havl, Nav3, Langham strain [16]) known to replicate in peritoneal macrophages from susceptible mice, but not from genetically resistant mice (5). Stock virus consisted of allantoic fluid with a hemagglutinin (HA) titer of 1:160 containing 10⁸⁷ mean tissue-culture infective doses per ml as titrated in susceptible A/J macrophages. NWS (H0, N1) was the Stuart-Harris strain of neurotropie influenza A virus (17). 10⁴ mean lethal doses (LD₅₀) (titrated in A/J mice) in 0.03 ml of A/J brain extract diluted in phosphate-buffered saline (PBS) were inoculated intracerebrally (i.c.) into ether-anesthetized mice as described (5). The human influenza strain HKH, (A/Hong-Kong/1/68; H3, N2), had undergone 132 passages in mouse lung (18); 0.1 ml of diluted A/J lung extract containing 100 LD₅₀ was inoculated intranasally (i.n.) into mice under ether anesthesia (6). TURH was the hepatotropic variant of avian influenza A/Turkey/England/63 (Havl, Nav3) causing acute liver necrosis and death when given intraperitoneally (i.p.) or intravenously (i.v.) (19). 0.2 ml of allantoic fluid diluted in PBS to contain 100 LD₅₀ (as estimated by i.v. or i.p. titrations in A/J mice) were given by either route as stated in the text.

HA titers were measured by standard procedures (6).

**Peritoneal Macrophage Cultures.** At various intervals after marrow grafting, macrophage cultures were established without killing the cell donor as previously described in detail (5). Briefly, mice were injected i.p. with 0.2 ml of 3% fluid thioglycolate medium (Difco Laboratories, Detroit, Mich.). 3 d later, peritoneal exudate cells were harvested and dishes containing 5 × 10⁶ macrophages were prepared from each individual mouse.

**Kupffer-Cell Cultures.** Nonparenchymal liver cells were isolated by 0.2% pronase digestion of the liver (pronase, B grade, Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) as described by Crofton et al. (20). The average yield was 2.1 × 10⁷ nonparenchymal cells per g of liver of both chimeric and normal control mice. 10⁷ nonparenchymal cells were cultivated on 21 × 26 mm cover glasses in Eagle's minimal essential medium (Gibco Diagnostics, Gibco Invenex Div., Glasgow, Scotland) supplemented with 20% heat-inactivated fetal calf serum (FCS), 100 IU/ml penicillin and 100 μg/ml streptomycin. Cultures were used 3 d later when >90% of adherent cells were Kupffer cells as judged by morphology and esterase staining (20) kindly performed by Dr. E. Müller, Institute of Anatomy, University of Zürich, Zürich, Switzerland.

**In Vitro Challenge with M-TUR.** Peritoneal macrophage and Kupffer-cell cultures were challenged as previously described (5) with egg-grown M-TUR virus at a multiplicity of...
infection (m.o.i.) of 10. Cytopathic effect (CPE) was scored by comparison with uninfected control cultures at the times after infection indicated in the text. HA titers of supernates were usually determined 48 h after infection.

**Immunofluorescence.** Indirect staining using mouse antisera prepared against A/Turkey/England/63, and fluorescein-isothiocyanate-conjugated rabbit anti-mouse-Ig antibodies were performed according to standard procedures as previously described (8).

### Results

**Generation of Susceptible or Resistant Peritoneal Macrophages in Chimeric Hosts.** Crosswise transfers of bone-marrow cells were made between susceptible A/J mice and resistant, Mx-heterozygous (A/J x A2G)F1 hybrid mice. Controls consisted of irradiated mice reconstituted with syngeneic marrow. Peritoneal exudate macrophages induced by thioglycolate medium were obtained from individual mice 12 wk after marrow grafting and were tested for susceptibility to infection with M-TUR virus in vitro. Table I shows that the peritoneal macrophages in these radiation chimeras exhibited the resistance phenotype of the bone-marrow-cell donor. Thus, the capacity of peritoneal macrophages to allow or restrict M-TUR replication in vitro was not influenced by factors of the host environment in which these cells differentiated, but represented a reliable genetic marker that was most useful for the verification of chimerism during the present study.

**Kupffer Cells from Chimeras Express Donor Phenotype In Vitro.** If the mononuclear phagocyte system were at all important for genetic resistance to viral infections, the fixed tissue macrophages located in the affected target organ would be expected to play a prominent role. Because myxovirus resistance is operative in the liver against infection with a hepatotropic virus strain (8), we tested Kupffer cells isolated from the livers of susceptible or genetically resistant adult mice in vitro. Similarly, using large challenge doses of M-TUR (m.o.i. = 10), we determined susceptibility of Kupffer-cell cultures established from individual chimeras. The results are shown in Fig. 1 and Table II. Kupffer cells obtained from susceptible A/J mice supported virus growth in the same manner as susceptible peritoneal macrophages. In contrast, Kupffer cells from resistant A2G mice showed no cytopathic effect and yielded no measurable HA. Chimeric mice used 12 and 44 wk after reconstitution gave Kupffer cells exhibiting the resistance phenotype of the bone-marrow donor. These data show that, under the present conditions, the bulk of hepatic macrophages was successfully replaced by cells derived from precursors present in the transplanted bone marrow.

**In Vivo Challenge of Chimeras Repopulated with Resistant or Susceptible Macrophages.** Because both free, and tissue, macrophage populations of susceptible mice had been successfully replaced by resistant macrophages and vice versa, the role mononuclear phagocytes might play in vivo could now be investigated. Individual radiation chimeras were checked for: (a) successful repopulation with resistant, or susceptible, peritoneal macrophages; and (b) susceptibility to in vivo challenge with TURH (19), a hepatotropic virus strain closely related to M-TUR. Table III summarizes the results: in vivo resistance reflected the genotype of the recipient host and not that of the donor macrophages. The susceptibility, or resistance, of the animal was independent of the type of macrophages present. These findings were confirmed in further experiments using a variety of influenza viruses given by different routes and affecting different organs (Table IV).

In order to deliberately expose the input virus to large numbers of resistant, or
MACROPHAGES AND INBORN RESISTANCE TO INFLUENZA VIRUSES

**Table I**

_Growth of M-TUR in Peritoneal Macrophages from Chimeric Mice In Vitro_

| Group | Recipient animals* | Bone-marrow cells§ | Number of animal | Growth of M-TUR in macrophages§ |
|-------|--------------------|---------------------|------------------|--------------------------------|
|       | Strain             | Genotype            | Donor strain     | CPE day | HA titer |
| 1     | A/J (+/+ )         | (A/J × A2G)F₁ (Mx/+ ) | 1    | - - | <1:2 |
| 2     | (A/J × A2G)F₁ (Mx/+ ) | A/J (+/+ )           | 5    | ++ ++  | 1:128 |
| 3     | A/J (+/+ )         | A/J (+/+ )           | 9    | ++ ++  | 1:128 |
| 4     | (A/J × A2G)F₁ (Mx/+ ) | (A/J × A2G)F₁ (Mx/+ ) | 13   | - - | <1:2 |

* Irradiated with 850 rad.
§ (A/J × A2G)F₁ hybrids are heterozygous for the dominant resistance gene Mx. A2G is a substrain of A/J and has the same H-2A (14).

Discussion

Peritoneal macrophages, but not other cell types in tissue culture, have so far been shown to mirror the genetic resistance of A2G mice in vitro (5). This could mean that the expression of the gene for myxovirus resistance might be confined exclusively to susceptible, macrophages, chimeric mice of both types, as well as normal control mice, were injected i.p. with fluid thioglycolate medium 3 d before challenge with 100 LD₅₀ of M-TUR virus by the same route. Even under these conditions, mortality was according to the genotype of the host and remained unaffected by the presence of induced macrophages of either type. Nevertheless, the course of the disease was slightly changed: susceptible chimeras harboring resistant macrophages died 16–24 h later than similarly treated susceptible control mice (on day 4 compared to day 3), suggesting that macrophages expressing their phenotype in vivo may have limited virus spread and multiplication. Similarly, susceptible macrophages may have supported virus growth in resistant mice because heterozygous Mx-carriers reconstituted with susceptible macrophages exhibited significantly higher serum-antibody titers specific for the HA of the infecting virus on day 7 after challenge than comparable controls (range 1:160–1:320 in the presence of susceptible macrophages compared to <1:10 in the presence of resistant macrophages).
Fig. 1. Kupffer cells from radiation chimeras exhibit the resistance phenotype of the bone-marrow donor. Kupffer cells were cultured for 3 d and then infected with M-TUR at a m.o.i. of 10. Note the morphological characteristics consisting of an indented or oval nucleus located eccentrically and the vacuolated cytoplasm (20). (A) and (B): Susceptible Kupffer cells isolated from control A/J liver, 6 h after infection. The same area is shown by phase contrast (A) and immunofluorescence microscopy (B). (C) and (D): Kupffer cells 48 h after infection prepared from 850-rad-irradiated chimeras 44 wk after reconstitution. Resistance of Kupffer cells from a susceptible (+/+ ) mouse repopulated with (Mx/+ ) bone marrow cells (C). Susceptibility of Kupffer cells from a resistant (Mx/+ ) mouse grafted with (+/+ ) bone marrow cells. Pronounced CPE consists in rounding of cells and detachment from the culture dish (D). ×840.
TABLE II

Growth of M-TUR in Kupffer Cells from Chimeric Mice In Vitro

| Group | Recipient animals* | Bone-marrow cells† | Number of animal | Time after reconstitution | Kupffer cells‡ |
|-------|--------------------|--------------------|-----------------|--------------------------|----------------|
|       | Strain Genotype    | Donor strain Genotype | Time | HA-titer, day | CPE, day 2 |
| 1     | (A/J (+/+))        | (A/J × A2GG)F1 (Mx/+) | 1    | 12 | <1:2 |
|       |                    |                    | 2    | 12 | <1:2 |
|       |                    |                    | 3    | 44 | <1:2 |
| 2     | (CBA × A2G)F1 (Mx/+)| (CBA × A/J)F1 (+/+)| 4    | 12 | +++ 1:32 |
|       |                    |                    | 5    | 12 | +++ 1:32 |
|       |                    |                    | 6    | 44 | +++ 1:32 |
|       |                    |                    | 7    | 44 | +++ 1:16 |
| 3     | (CBA × A/J)F1 (+/+)| (CBA × A/J)F1 (+/+)| 8    | 12 | +++ 1:32 |
| 4     | (CBA × A2G)F1 (Mx/+)| (CBA × A2G)F1 (Mx/+)| 9    | 12 | <1:2 |
| 5     | A/J controls (+/+)| — — | 10 | — | +++ 1:32 |
|       |                    |                    | 11 | — | +++ 1:32 |
| 6     | A2G controls (Mx/Mx)| — — | 12 | — | <1:2 |
|       |                    |                    | 13 | — | <1:2 |

* Irradiated with 850 rad (group 1) or 950 rad (groups 2-4).
† As in Table I. CBA/J are H-2k.
‡ 3-d cultures were infected with M-TUR at a m.o.i. of 10.

the population of macrophages. Alternatively, virtually all cells of the body might be able to express the resistance gene in vivo but, with the exception of macrophages, would be incapable of realizing this faculty under tissue-culture conditions. The present data show that cultured Kupffer cells isolated from the liver of untreated, adult mice behave exactly in the same manner as peritoneal macrophages. Thus, the resident macrophage population of the liver would seem to display, in addition to most other well known characteristics of mononuclear phagocytes (20), the same resistance properties as peritoneal exudate macrophages.

Previous work on macrophage origin in mouse radiation chimeras were based on differences in karyotypes (10, 13) or differences in cell-surface antigens (11, 12) between donor, and host, cells. To our knowledge, differences in genetically determined antiviral functions have never been used as markers in such studies. It was therefore not a priori evident that, with regard to virus susceptibility or resistance, macrophages in chimeric mice would express the phenotype of the grafted precursor cells from which they were derived. The present results are clear-cut: generation of resistant or susceptible macrophages was an autonomous function of the transplanted bone-marrow stem cells, and was not influenced by factors of the host environment.

In contrast to chromosome-marker techniques widely used in repopulation experiments (10, 13), testing of Mx-gene expression is not restricted to dividing cells. Hence, our results on bone-marrow origin of Kupffer cells are most likely representative for the entire liver macrophage population. The present data using a functional assay demonstrate that bone-marrow transplantation in mice results in a repopulation of the tissue-macrophage compartment with marrow-derived donor cells. They corroborate previous findings in rodents (12, 13) as well as in man (21).
**Table III**

*In Vivo Resistance of Chimeras Reconstituted with Bone Marrow from Susceptible or Resistant Donors to Infection with TURH*

| Experiment | Group | Challenged animals* | Bone-marrow cell† | Resistance of § macrophage cultures | Survived II |
|------------|-------|---------------------|-------------------|------------------------------------|------------|
| A          | 1     | A/J (+/+)           | (A/J × A2G)F₁ (Mₓ/+)| 10/10                              | 0/10       |
| A          | 2     | (A/J × A2G)F₁ (Mₓ/+)| A/J (+/+)         | 0/8                                | 8/8        |
| A          | 3     | A/J (+/+)           | A/J (+/+)         | 0/8                                | 0/8        |
| A          | 4     | (A/J × A2G)F₁ (Mₓ/+)| (A/J × A2G)F₁ (Mₓ/+)| 10/10                              | 10/10      |
| B          | 5     | CBA/J (+/+)         | (CBA/J × A2G)F₁ (Mₓ/+)| 4/4                                | 0/4        |
| B          | 6     | CBA/J (+/+)         | CBA/J (+/+)       | 0/4                                | 0/4        |
| C          | 7     | (CBA/J × A2G)F₁ (Mₓ/+)| (CBA/J × A/J)F₁ (+/+) | 0/4                                | 4/4        |
| C          | 8     | (CBA/J × A2G)F₁ (Mₓ/+)| (CBA/J × A2G)F₁ (Mₓ/+)| 4/4                                | 4/4        |

* Recipients were irradiated with 850 rad (experiments A, B) or 950 rad (Experiment C).
† As in Table I, CBA/J are H-2K.
§ Macrophage cultures were established 4 wk (experiment B), 12 wk (experiment A), or 24 wk (experiment C) after marrow grafting. Number of resistant cultures/total number of macrophage cultures tested.
II Macrophage donors were allowed to recover for 14 d and were then challenged i.p. with 100 LD₅₀ of TURH. Number of survivors on day 7/total number of mice inoculated.

Therefore, if macrophages were instrumental for in vivo resistance, the livers of susceptible mice harboring resistant Kupffer cells should have resisted infection with the hepatotropic influenza virus strain TURH. M-TUR used for the classification of macrophage susceptibility in vitro is intimately related to TURH from which it has been derived (5). Our evidence that macrophage populations classified as genetically resistant or susceptible in vitro did in fact express their phenotype in vivo is, of necessity, indirect and rests on the following findings: (a) immunofluorescent staining of liver sections from TURH-infected chimeras revealed extensive Kupffer-cell fluorescence in preparations from animals reconstituted with susceptible macrophage precursors, but not in preparations from mice grafted with bone marrow of resistant donors (not shown); (b) survival time after M-TUR infection of susceptible chimeras repopulated with resistant macrophages was somewhat prolonged in comparison to that of similarly infected susceptible control animals; (c) virus-specific-antibody titers were significantly higher in resistant mice harboring susceptible macrophage populations than in comparable controls, possibly reflecting virus growth in graft-derived cells. Nevertheless, the replacement of resistant macrophages by susceptible ones did not render genetically resistant hosts susceptible. Despite complete repopulation of susceptible mice with a resistant macrophage system, the overall susceptibility of the animals was preserved. It can therefore be concluded that the genetic resistance of A2G mice is not due to a selective resistance of their macrophages. The recent claim of Virelizier and collaborators (22) that the unique behavior of A2G macrophages was responsible for in vivo resistance of this mouse strain was deduced primarily from the striking correlation between in vitro macrophage resistance and resistance of A2G mice in vivo. It should be reconsidered in the light of the present repopulation experiments demonstrating an obvious disparity between macrophage susceptibility and susceptibility of the chimera from which the macrophages were obtained. Further
TABLE IV

| Virus Strain | Group | Challenged animal | Bone-marrow cell§ | Resistance of macrophage cultures¶ | Survivors¶ |
|--------------|-------|------------------|-------------------|-----------------------------------|------------|
| NWS; i.c.    | 1     | (A/J x A2G)F1    | A/J               | (+/+)                             | 0/3        |
|             | 2     | CBA/J (+/+)      | (CBA/J x A2G)F1  | (Mx/+)                            | 4/4        |
|             | 3     | (CBA/J x A2G)F1 | (Mx/+)            | CBA/J (+/+)                       | 0/4        |
|             | 4     | CBA/J (+/+)      | CBA/J (+/+)       |                                   | 0/4        |
| HKH; i.n.   | 5     | A/J (+/+)        | (A/J x A2G)F1    | (Mx/+)                            | 5/5        |
|             | 6     | (CBA/J x A2G)F1 | (Mx/+)            | CBA/J (+/+)                       | 0/5        |
|             | 7     | (CBA/J x A2G)F1 | (+/+) Controls    |                                   | 0/5        |
|             | 8     | (CBA/J x A2G)F1 | (Mx/+) Controls   |                                   | 5/5        |
| TURH; i.v.  | 9     | (A/J x A2G)F1   | A/J               | (+/+)                             | 0/6        |
|             | 10    | A/J (+/+)        | (+/+) Controls    |                                   | 0/6        |
|             | 11    | (A/J x A2G)F1   | (Mx/+) Controls   |                                   | 0/6        |

* Virus challenge was performed in mice from which macrophages had been obtained 2 wk previously as described in Materials and Methods.
† As in Tables I and II, 950 rad was used.
§ See Tables I and II.
¶ Peritoneal macrophages obtained individually from thioglycolate-stimulated mice were classified as resistant or susceptible to M-TUR. Number of resistant cultures/total number of macrophage cultures tested.
¶ Number of survivors on day 14/total number of mice challenged.

evidence against a decisive role of Kupffer cells in mediating inborn resistance in the liver, stems from the finding that circumventing this hypothetical first barrier by injection of TURH virus directly into the bile duct resulted in self-limiting hepatocyte lesions identical to those usually observed (8, 23) after i.p. or i.v. virus injection (H. Arnheiter, unpublished results). Moreover, resistance of A2G mice has been found to be preserved despite in vivo treatment with inhibitors of macrophage function such as silica or thorium dioxide (8).

In conclusion, macrophage resistance (as assessed in vitro) and in vivo resistance of the animal against influenza virus, both brought about by the gene Mx, do not seem to be causally related. We have, therefore, to assume that, in vivo, a resistance mechanism governed by the dominant allele Mx, and possibly very similar to that found in macrophages, has to be operative in most, if not all, cells throughout the body.

The present findings might apply to other virus-host systems in which a prominent in vivo role of macrophages exhibiting genetically determined resistance in vitro has been postulated (3, 4, 24, 25). In most instances, additional host defense mechanisms seem to be involved; e.g., thymus-dependent, cell-mediated immune responses in C3H mice genetically resistant to mouse hepatitis virus-2 infection (26), natural killer-cell-like activity in resistance of C57BL/6J mice to infection with herpes simplex virus-1 (27), and interferon in the case of the genetic resistance of mice to mouse hepatitis virus-3 (28). Recent evidence from our laboratory indicates that interferon is also an important factor in myxovirus resistance, because i.v. administration of sheep antimouse-interferon globulin rendered resistant mice susceptible to the lethal effect of various influenza virus strains (29). How interferon cooperates with the gene Mx to create a resistance state that is highly specific for a group of closely related viruses is still far from clear. Whatever the resistance mechanisms may eventually turn out to
be, this interferon-dependent inborn resistance is preserved in radiation chimeras and is, as revealed by the present experiments, independent of Mx-gene expression in cells of the hemopoietic system.

Summary

Radiation chimeras produced by croswise transfers of bone-marrow cells among histocompatible mice susceptible, or genetically resistant, to lethal challenge by a number of myxoviruses were used to test whether macrophage resistance (as assessed in vitro) and resistance of the animal (as measured in vivo), both previously shown to be brought about by the gene Mx, were causally related.

49 chimeras were tested individually, both for resistance of their macrophages to in vitro challenge with M-TUR (a strain of avian influenza virus A/Turkey/England/63 adapted to grow in cultured mouse peritoneal macrophages), and for resistance of the animal in vivo upon challenge with pneumotropic, neurotropic, or hepatotropic influenza viruses. Cultivated Kupffer cells and peritoneal macrophages harvested from chimeric mice expressed the resistance phenotype of the bone-marrow donor irrespective of the host environment in which they had differentiated. However, susceptibility or resistance in vivo was according to the genotype of the host. Thus, inborn resistance of radiation chimeras was found to be independent of Mx-gene expression in cells of the hemopoietic system.

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