INBORN RESISTANCE OF MICE TO MYXOVIRUSES: MACROPHAGES EXPRESS PHENOTYPE IN VITRO

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Compared with acquired immunity which is being so extensively studied, genetically determined or inborn resistance to infectious agents is poorly understood. Promising murine model infections exist in which single gene inheritance has been well documented (1). A good example is the resistance to the lethal effects of various myxoviruses exhibited by mice carrying the dominant allele *Mx* (2, 3). This resistance is operative against neurotropic influenza viruses injected intracerebrally, pneumotropic strains injected intranasally, and a hepatotropic strain injected intraperitoneally (4). Experiments in vitro on tissue cultures with appropriately adapted virus strains gave either ambiguous results or showed that fibroblasts, kidney cells, and nerve cells from resistant and susceptible animals were comparable in their ability to support virus replication and to suffer cytopathic damage (3, 5, unpublished observations).

The capacity of peritoneal macrophages to express virus resistance in vitro has been observed in several systems (6, 7). Mouse-adapted strains of influenza virus do seem to replicate in macrophages, but only at low levels and in a small percentage of cells (8). A distinction between resistance and susceptibility based on control values of only borderline significance would be very fastidious. However, a strain of avian influenza virus which proved exceptionally flexible, and which had been adapted to mouse kidney cells in vitro, to Ehrlich ascites tumor cells and to mouse liver (9), eventually evolved the capacity to cause a marked cytopathic effect in macrophages of susceptible animals (J. L. Virelizier, 1974, personal communication). We therefore decided to further adapt this strain so that it would replicate reliably in mouse peritoneal macrophages in vitro, thus allowing unequivocal classification of mice according to the susceptibility of their macrophages.

If resistance of macrophages in vitro and resistance of the whole animal were governed by different genes, these should segregate in backcross animals. To test for this possibility, we determined macrophage susceptibility individually and, in the same animals, resistance to intracerebral challenge of backcross mice. We now report that all backcross mice whose macrophages had been classified as susceptible in vitro succumbed to intracerebral challenge with a neurotropic influenza A virus, whereas the great majority of mice with resistant macrophages survived challenge.

Materials and Methods

Mice. Inbred A2G mice, homozygous for the resistance allele *Mx* (2) were bred locally from nuclei obtained from the Laboratory Animals Centre, Carshalton, Surrey, England. A/J mice were purchased from the Jackson Laboratory, Bar Harbor, Maine. Crosses (A2G × A/J)F_1 and backcrosses (F_1 × A/J) were arranged in our laboratory. BALB/cJ, CBA/J, C3H/J, C57BL/6J, DBA/2J, SB/Le (originally a gift from Dr. P. W. Lane of The Jackson Laboratory), and various F_1 hybrids between A2G and these strains were bred locally.

Viruses. Avian influenza A virus, strain M-TUR, was derived from A/Turkey/England/63 (Hav1Nav3, Langham strain) (10) as described in the text. Human influenza A virus, strain NWS (HoN1), was the neurotropic variant of Stuart-Harris (11).

Virus Titrations. These were done by standard procedures (12). Infectivity of M-TUR was titrated by intra-allantoic inoculation of serial 10-fold virus dilutions into 10-day-old embryonated eggs. NWS was assayed by intracerebral titration in adult A/J mice. Hemagglutinin and hemagglutination-inhibition titers were measured by the pattern method in World Health Organization (WHO) hemagglutination trays. Identity of the viruses was repeatedly checked by hemagglutination-inhibition with rabbit and chicken antisera prepared several years earlier against A/Turkey/England/63 and NWS, the two strains originally obtained from the World Influenza Centre, Mill Hill, England.

Media. Buffered saline contained 8.0 g NaCl, 2.7 g $Na_2HPO_4 \cdot 7H_2O$ and 0.4 g KH_2PO_4/litre. Culture medium consisted of 70% RPMI-1640 with L-glutamine (Gibco Bio-Cult, Glasgow, Scotland), 30% fetal calf serum (Serva Feinbiochemica, Heidelberg, West Germany), penicillin (100 U/ml), and streptomycin (100 μg/ml).

Macrophage Cultures. Batches of 10–14 backcross mice together with 3 (A2G × A/J)F_1 (resistant controls) and 3 A/J (susceptible controls) of either sex, aged 8–12 wk, were stimulated by 2-ml intraperitoneal injections of fluid thioglycollate medium (Difco Laboratories, Detroit, Mich.) on day 3. On day 0, the peritoneal cavity of each mouse held under ether anesthesia was rinsed by injecting 10 ml of buffered saline containing 200 U/ml of penicillin and 200 μg/ml of streptomycin, and withdrawing from it as much fluid as possible. Usually, ≅5 ml of fluid containing between 2 and 4 × 10^6 nucleated cells (as estimated from hemocytometer counts) could be recuperated. These cells were rapidly chilled and were washed twice in buffered saline with antibiotics by centrifugation at 150 *g* for 8 min in a refrigerated horizontal centrifuge. Cell harvests from each individual mouse were resuspended in 4 ml of culture medium and were distributed equally into two 35-mm diameter wells of FB-6-TC disposable six-well tissue culture trays (Linbro Chemical Co., Hamden, Conn.). (Plates with smaller wells had proved less suitable.) The plates were incubated at 37°C in an atmosphere of 5% CO_2 in air. After 3–4 h, the plates were thoroughly agitated by hand to resuspend all cells not firmly attached, the fluid was withdrawn and replaced with 1.5 ml of culture medium, and the plates were returned to the incubator. On day +1 the plates were again agitated and the fluid was replaced with 1.5 ml of fresh culture medium. The cells were then ready for virus challenge. With some practice, an overall failure rate of <10% could be maintained throughout the experiments to be reported. Failures included the following: death of the animal during anesthesia, upon removal of peritoneal fluid or shortly thereafter; low yield of macrophages, so that only scattered cells settled in the wells, leaving large empty spaces; and bacterial contamination of macrophage cultures. After withdrawal of peritoneal macrophages as described above, mice were rested for at least 2 wk before being subjected to in vivo challenge.

In preliminary experiments in which survival of the macrophage donor was not essential, the animals were killed and the peritoneal cavities were opened for rinsing. At least twice as many cells could be recuperated by this procedure.

Virus Challenge in Vitro. 0.05 ml of M-TUR virus seed (tissue culture fluid from the 20th in vitro A/J macrophage passage), containing 10^8 50% egg infecting doses (EID50)[1] per ml, was added to one of each pair of wells containing macrophages of individual backcross or control mice prepared as described above. 48 h after challenge, the wells were inspected with an inverse phase contrast microscope (× 40 objective) and the occurrence of a cytopathic effect was scored by comparison with the uninfected control well. This scoring was quite unequivocal when reasonable

[1] *Abbreviations used in this paper:* EID50, 50% egg infecting dose.

numbers of macrophages were present in each field, but it proved impossible in very sparsely seeded wells. Such macrophage cultures were registered as failures, as were cultures with bacterial contamination. The 48-h culture fluids were removed and titrated individually for hemagglutinin as an additional check. All wells with cytopathic effect yielded fluids with hemagglutinin titers >1:256, whereas wells without obvious cytopathic effect had titers <1:64 and frequently contained no measurable hemagglutinin. This correlation between hemagglutinin titers and the occurrence of cytopathic effects was absolute, again disregarding wells containing only very few macrophages. Macrophages from A/J mice (susceptible controls) and from (A2G × A)F_1 mice (resistant controls), included in each series of backcross mice tested, always behaved as expected.

Virus Challenge in Vivo. NWS virus, kept as 10% A/J brain extract and diluted to contain 1,000 mean lethal doses in 0.03 ml of buffered saline as estimated from titration in A/J mice, was inoculated intracerebrally into mice under ether anesthesia and from which macrophages had been obtained 2 or more wk previously. Deaths occurring within 24 h of injection (accounting for never more than 10% of injected animals) were discounted as traumatic. Deaths occurring from the 3rd to the 8th day after inoculation were scored as the result of neurotropic influenza infection. Since in previous larger series, deaths beyond the 8th day rarely occurred, animals were observed for 15 days only; those surviving this interval were classified as resistant. Resistant and susceptible controls were always included.

Results

Adaptation of A/Turkey/England/63 to Susceptible Mouse Peritoneal Macrophages. As noticed by J. L. Virelizier (1974, personal communication), a strain of avian influenza A virus which we had previously adapted to mouse kidney cells and to Ehrlich ascites tumor cells induced cytopathic effects in mouse peritoneal macrophages. The growth of this virus in macrophage cultures was rather irregular. We observed later that the same strain, after further adaptation to mouse liver (9), sometimes reached high hemagglutinin titers in the mouse peritoneal cavity. Starting from this liver-adapted virus, we performed a number of rapid (12–24 h) passages in vivo from peritoneum to peritoneum. After six such in-vivo passages it was possible to pass the virus serially in susceptible macrophage cultures in vitro. No difficulties were encountered, and from the 17th to the 20th in-vitro passage in A/J macrophages the properties of the virus remained stable. Most work was done with virus taken from the 20th passage. This macrophage-adapted variant will be called M-TUR.

M-TUR was able to grow in the allantoic cavity of chick embryos, and egg infectivity to hemagglutinin ratios remained around 10^6 during the entire passage series. The virus produced plaques in chick embryo fibroblast monolayers. Rabbit immune serum and chick convalescent serum prepared many years earlier inhibited hemagglutination and plaque formation of M-TUR, and of the original strain to the same high titer.

Comparative Growth of M-TUR in Macrophages from A/J and A2G Mice. Fig. 1 illustrates the growth of M-TUR in macrophages from a susceptible strain (A/J) and in macrophages from a resistant strain, homozygous for the allele *Mx* (A2G). No evidence of replication was seen in A2G macrophages, whereas rapid and extensive growth occurred in A/J macrophages.

The difference between resistant and susceptible macrophages was also measurable by hemagglutinin: depending upon the input virus dose, A2G macrophages either yielded no measurable hemagglutinin or low levels only.

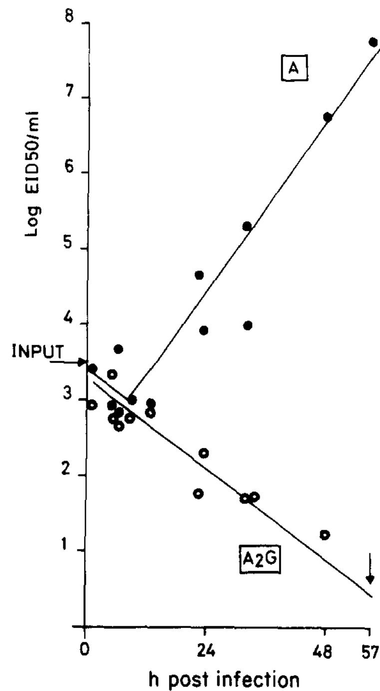

FIG. 1. Growth curves of M-TUR virus in macrophages from susceptible A/J mice (A) and in macrophages from homozygous Mx-bearing mice (A2G). Input virus was not removed. Each point represents the contents of one well.

Susceptible macrophages produced sufficient extracellular virus within 48–96 h of incubation to reach hemagglutinin levels of 1:256 and higher. Macrophages obtained from mice not stimulated with thioglycollate reacted similarly to those harvested by the standard procedure. It is possible that low level hemagglutinin in resistant cultures was the result of a variable degree of fibroblast contamination.

Cytopathic effects of M-TUR in Macrophages from Susceptible and Resistant Mice. 48 h after a rather large challenge dose of M-TUR (5×10^6 EID_{50}), A/J macrophages showed a very pronounced cytopathic effect (Figs. 2, 3). The macrophages were rounded and their outline was blurred. To fully appreciate this effect, the cultures had to be viewed with a $\times$ 40 phase contrast objective, since rounding and clumping of cells without blurring of the outlines sometimes occurred in uninfected control cultures or in infected resistant cultures. After very small challenge doses, the cytopathic effect took 24–48 h longer to develop. After very large doses of either M-TUR or other influenza A viruses (of the order of 100–1,000 hemagglutinating units/well) a cytopathic effect, probably toxic in nature and affecting A2G and A/J macrophages equally, was observed. In A2G macrophages this effect was not transmissible in series.

M-TUR proved cytopathic with small variations in timing for macrophages of the following strains: BALB/cJ, CBA/J, C3H/J, C57BL/6J, DBA/2J, and SB/Le. Cytopathic effects, excepting the toxic manifestation alluded to above, were

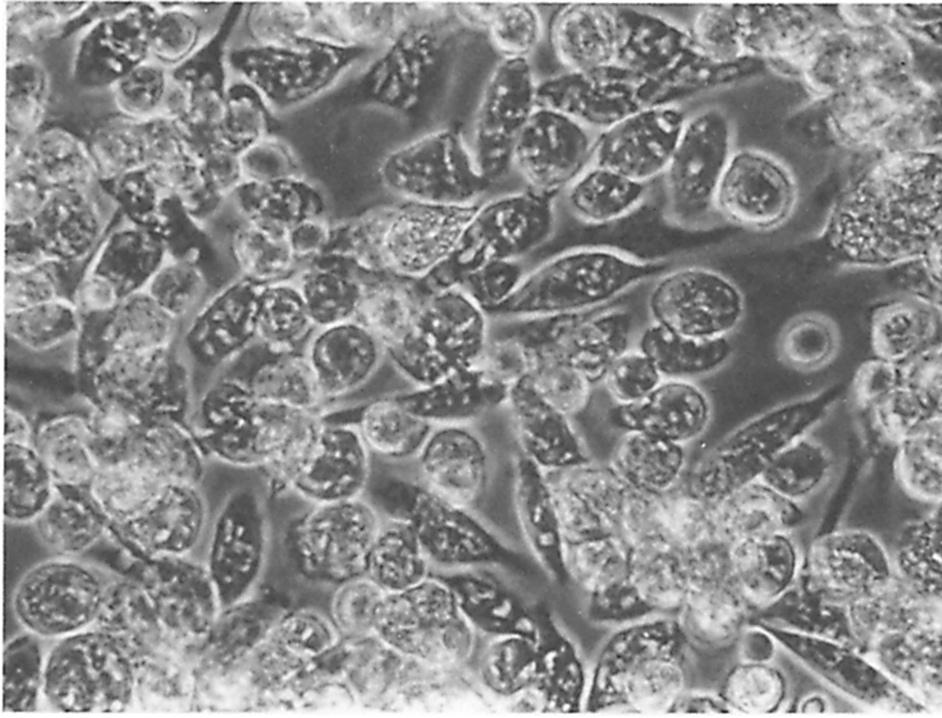

FIG. 2. A2G macrophages 48 h after infection with 5×10^6 EID50 of M-TUR. Uninfected susceptible or resistant macrophages showed a very similar picture. Magnification × 850.

never seen in macrophages from A2G mice or from (A2G × A)F_1, (A2G × CBA)F_1, (A2G × C57BL/6)F_1 or (A2G × SB)F_1 hybrids.

Failure to Adapt M-TUR to Resistant Macrophages. Several useful experiments might have been performed if it had been possible to produce a variant of M-TUR capable of overcoming whatever barrier to its growth the allele *Mx* opposes. Straightforward serial passages in A2G macrophages resulted in rapid loss of the virus. Criss-cross passages between resistant and susceptible macrophages could be carried out for prolonged periods, but no evidence of increased growth potential in resistant macrophages was obtained. In mixtures of susceptible and resistant (either A2G or F_1) macrophages the virus grew, but no adaptation to the resistant cells was achieved.

Resistance of Macrophages and of Whole Animals in Offspring from Backcross Experiments. Since macrophages could be obtained and tested without sacrificing the cell donor, the following experiment was performed. (A2G × A/J)F_1 mice were backcrossed to the susceptible parent. From previous data (2, 3) we could expect 50% of these backcross mice to prove resistant when challenged via the intracerebral route with the neurotropic influenza A virus NWS. Macrophages from individual backcross mice were challenged in vitro with M-TUR and classified as either resistant or susceptible (intermediate or doubtful results were not obtained when we adhered to the conditions described in Materials and Methods). The same mice were later challenged intracerebrally

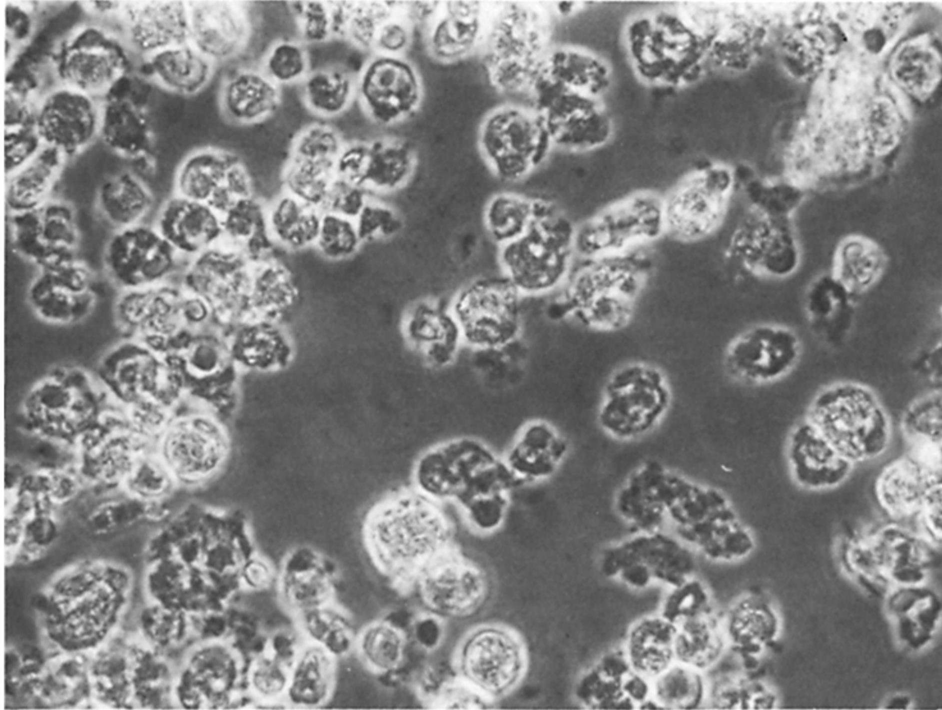

FIG. 3. A/J macrophages 48 h after infection with 5×10^6 EID50 of M-TUR. Cytopathic effect consists in rounding of cells, blebbing and blurring of cellular contours. Magnification × 850.

TABLE I

*Resistance of Macrophages and Resistance to Intracerebral Challenge of Backcross Mice Segregating for the Allele Mx**

	Result of challenge in vivo‡		
	Survived	Died	Total
Macrophages§ resistant	26	2	28
Macrophages susceptible	0	36	36
Total	26	38	64

* (A2G × A/J)F_1 were backcrossed with A/J. The offspring were individually tested for macrophage resistance and for resistance to intracerebral challenge.

‡ Mice whose macrophages had been previously classified as either resistant or susceptible were challenged by intracerebral inoculation of NWS (Materials and Methods).

§ Peritoneal macrophages were obtained from individual mice and challenged with M-TUR (Materials and Methods).

with NWS, and their death or survival was noted. The results of this experiment which was performed successfully on a total of 64 animals, are shown in Table I.

Macrophages from 28 mice were classified as resistant, and those from 36 as

susceptible (this is compatible with the 1:1 ratio expected from a one-gene hypothesis). Judging from death and survival, 26 were classified as resistant and 38 as susceptible (this is still compatible with a 1:1 ratio). All 36 mice with susceptible macrophages died. Of the 28 mice with resistant macrophages, 2 died. One of these died on the 8th day; and although such late deaths sometimes occur in susceptible animals, they are rare and would not be expected in a small series. The other mouse died on the 5th day, which is the peak time for deaths in susceptible mice. The exact cause of death in these two mice could not be established.

Discussion

Adaptation of a strain of avian influenza virus to mouse macrophages proved relatively easy, no doubt because the potentiality for inducing cytopathic damage in macrophages was already inherent in the virus we had grown in Ehrlich ascites cells and later in liver (4). It is impossible to guess at what time this property developed. Growth of fowl plague virus in chicken macrophages has been reported (13). The derivation of a strain adapted to mouse macrophages directly from an original avian isolate has not been attempted, but might be successful. Part of the adaptation process probably consists of the ability to grow in the presence of large amounts (30%) of fetal calf serum which is inhibitory for most influenza virus strains.

Although the resistance pattern of macrophages from different strains of mice and from F_1 crosses between resistant (A2G) and susceptible animals made it likely that resistance in vivo as described earlier (2, 3, 4, 12) and the present observations on macrophage resistance in vitro were two facets of the same phenomenon, it seemed important to provide additional arguments in favor of a unitarian concept. This was necessary, moreover, since the two viruses used, M-TUR for the experiments on macrophages in vitro, and NWS for challenge in vivo, are probably as far from each other as any two influenza A viruses can be. Furthermore, it might have been argued that the various adaptation processes to which M-TUR had been subjected resulted in a mere artifact with little bearing on real life situations.

The simplest approach, inspired by earlier experiments of Kantoch et al. (14) on resistance to mouse hepatitis virus, was to check in suitable backcrosses whether or not the two properties would co-segregate. Had we been able to find mice with susceptible macrophages but surviving virus challenge, we might, by further breeding, have delineated a particular in vivo resistance factor. We did not encounter such animals. On the other hand, we did observe two mice which died, although their macrophages had been classified as resistant. For this point to be definitely settled, one would have to first obtain litters from a large number of backcross mice and then repeat an experiment of the type just described on the parents of these litters. For the time being we prefer to attribute these two deaths to intercurrent causes and to regard the two forms of resistance as exact correlates of each other, as has been found with mouse hepatitis (15).

If resistance of macrophages and of the whole animal are correlated, it would be tempting to view them as causally related. Macrophages are ubiquitous elements. This could explain why organs of such histological diversity as lung

and liver both display resistance (4). To explain resistance of the brain (16) one could invoke the presence of some macrophage analogue in nervous tissue. Attributing resistance solely to the macrophages would also be compatible with the finding that other cells of the body, when tested in tissue culture, do not seem to express resistance, at least not to the same degree (3, 5, unpublished observations). However, cells in tissue culture do not behave in the same manner towards virus infections as do cells within organs. Whereas macrophages after removal from the peritoneal cavity appear to keep their typical characteristics for some time and do not divide, kidney cells or fibroblasts suffer a great deal of de-differentiation. In contrast to adult mice, newborn *Mx*-bearing animals are susceptible (2). Tissue culture might involve regression to a phase before the maturation step needed to express resistance.

In artificial mixtures of resistant and susceptible macrophages in vitro, virus replication was depressed below the level expected from the number of susceptible cells present (unpublished observations). This might indicate some protective effect of resistant macrophages and could be viewed as a model of what happens in vivo. We do not feel confident enough to draw definitive conclusions, since the resistant macrophages in artificial mixtures might simply act as a sort of virus sink. Clarification of the role of macrophages in vivo must await reconstruction experiments in which macrophage populations of susceptible mice will be replaced by resistant macrophages and vice versa.

Whether macrophages are instrumental in bringing about resistance of the whole animal or whether they simply express in vitro a phenomenon which other body cells express in vivo only, there is little doubt that the mechanism involved at the cellular level must be very similar. It would be far-fetched indeed to assume that the same gene brings about the same result by different means. Hence, if the exact step at which virus infection is arrested in macrophages could be elucidated, our understanding of the resistance induced by the allele *Mx* would be much advanced. The molecular biology of myxoviruses is known in such detail that sizable progress should be within reach. Resistance is caused by the presence of one dominant allele, which must govern the production of one gene product directly or indirectly responsible for resistance. The mechanisms involved should be relatively simple, with a point of attack common to most myxoviruses. The block attributable to Mx is not easily circumvented, since we were unable to adapt a virus to grow in resistant macrophages. All these considerations suggest that clarification of inborn resistance to myxoviruses, which now can be pursued in macrophage cultures rather than in whole animals, may be highly rewarding.

Summary

A strain of avian influenza A virus was adapted to grow in mouse peritoneal macrophages in vitro. The adapted strain, called M-TUR, induced a marked cytopathic effect in macrophages from susceptible mice. Mice homozygous (A2G) or heterozygous (F_1 hybrids between A2G and several susceptible strains) for the gene *Mx*, shown previously to induce a high level of resistance towards lethal challenge by a number of myxoviruses in vivo, yielded peritoneal

macrophages which were not affected by M-TUR. Peritoneal macrophages could be classified as resistant or susceptible to M-TUR without sacrificing the cell donor.

Backcrosses were arranged between (A2G × A/J)F_1 and A/J mice. 64 backcross animals could be tested individually both for resistance of their macrophages in vitro after challenge with M-TUR, and for resistance of the whole animal in vivo after challenge with NWS (a neurotropic variant of human influenza A virus). Macrophages from 36 backcross mice were classified as susceptible, and all of these mice died after challenge. Macrophages from 28 mice were classified as resistant, and 26 mice survived challenge. We conclude that resistance of macrophages and resistance of the whole animal are two facets of the same phenomenon.

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