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*MOUSE MACROPHAGES AS HOST CELLS FOR THE MOUSE HEPATITIS VIRUS AND THE GENETIC BASIS OF THEIR SUSCEPTIBILITY**

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Communicated by Bentley Glass, June 20, 1960

Much of the mystery of pathogenesis of animal virus disease lies in the realm of specificity of virus effect on cell type and in the differing effect of a given virus on different hosts. Tissue culture, despite its great contributions to other aspects of cytopathology, has not so far furnished significant clues to these basic problems. We have recently found that an acute virus disease of mice, mouse hepatitis,[1, 2] has a selective destructive effect for cells derived from the reticulo-endothelial system (macrophages) and that the apparent genetic difference in susceptibility of different strains of mice is reflected in the behavior of macrophages from these strains in tissue culture. Tests of hybrids resulting from crosses between resistant and susceptible strains indicate that susceptibility is inherited and that genetic segregation of susceptibility and resistance occurs in the F_2 and backcross generations.

Materials and Methods.—The macrophages were obtained by explanting fragments of liver from newborn (1–3 days old) mice into roller tubes either directly onto the glass or onto a reconstituted collagen substrate.[3, 4] The collagen was prepared according to the method of Ehrmann and Gey by extraction of 0.1 per cent acetic acid, dialysis against distilled water, and reconstitution to an agar-like slant with ammonium hydroxide vapors. The supernatant medium, except in otherwise specified cases; consisted of 60 per cent Gey's balanced salt solution, 10 per cent chick embryo extract (50 per cent), and 30 per cent horse serum (obtained from Microbiological Associates), with 0.004 per cent phenol red, 100 units of penicillin, and 10 micrograms of streptomycin. In some experiments, as designated in the text, chicken serum or a combination of chicken serum and horse serum was used. The chicken serum was obtained from White Leghorns kept for routine bleeding in our laboratory. The cultures were incubated in a roller drum and maintained in this manner at 37°C throughout the experiments.

The cultures were inoculated with the virus three to four days following explantation. The medium was renewed every two to three days until the end of the particular experiment. Supernatant fluids were frozen at −40°C and reserved for titration in mice. The cells were observed directly in the roller tube and their

appearance was recorded. Photographic records were obtained at intervals throughout the experiments.

The mouse hepatitis virus used in our laboratory is a virulent strain originally obtained from Dr. John Nelson of The Rockefeller Institute. Ten per cent stock liver suspensions were maintained by serial passages into weanling Swiss mice. Tissue culture supernatant fluids were routinely tested by inoculation into weanling mice of Princeton strain (Pr). Autopsy of survivors showed that an occasional surviving mouse had specific lesions, so that virus was patently present even though the mouse survived. Our titrations include these animals, therefore, but in no case was the final end point increased by as much as one log dilution. Several other strains of mice were tested briefly and their use is mentioned in the text. The method of crossbreeding is also indicated in the text.

Results.—In our early attempts to obtain cell destruction we studied the effect of the virus on parenchymal liver cells obtained from mice of the Pr strain. Various media were tested in an attempt to culture these cells. First, human, rabbit, and horse sera, with beef embryo extract, were used. In these media outgrowth consisted mainly of large, granular, single cells with very little organized formation. Virus was recovered from some of the inoculated cultures, but not consistently, nor did the virus persist, nor were significant destructive effects produced.

When chick embryo extract was substituted, and horse and chicken sera used, outgrowths consisted of pavement-like epithelium with fibroblasts interspersed throughout, and excellent cords of granular liver cells were found developing from the edge of some explants (Fig. 2). After studying conditions under which such organized liver cell growth occurred in the roller tube primary explants (age of the mouse, various supplements to the media, the area of the liver from which the explant was obtained), it was found that the appearance of liver cords was related to the presence of chicken serum in the medium. This was true, however, only in the absence of collagen. The cultures were inoculated with mouse hepatitis and virus was recovered from the cultures with healthy liver cords as long as twelve days after inoculation. However, no destruction of liver cells was observed.

Two factors then brought to light the macrophage susceptibility which had not been observed previously. First, in the electron microscope, large amounts of apparent virus were visible on the inner side of the endothelial cells lining the liver sinusoids (Fig. 1), and secondly, when the liver was cultured on reconstituted collagen, great numbers of macrophages were obtained. Macrophages were first seen in cultures of mouse liver grown on collagen in a medium containing 20 per cent horse serum. Three days following inoculation of the virus the control cultures had a heavy concentration of macrophages, yet no macrophages remained in the virus-inoculated cultures. Virus was recovered from the inoculated cultures as late as 20 days following inoculation. It was subsequently found that 30 per cent horse serum yielded better macrophages, and this medium was used throughout the rest of the experiments.

The original colony outgrowths consisted of clear, pavementlike epithelium, over which fingers and sheets of liver parenchymal cells grew, with fibroblasts interspersed throughout the colony area (Figs. 3 and 4). Large numbers of macrophages migrated away from the original colony, and to the side of the tube opposite to the explants. The macrophages were readily identifiable by their

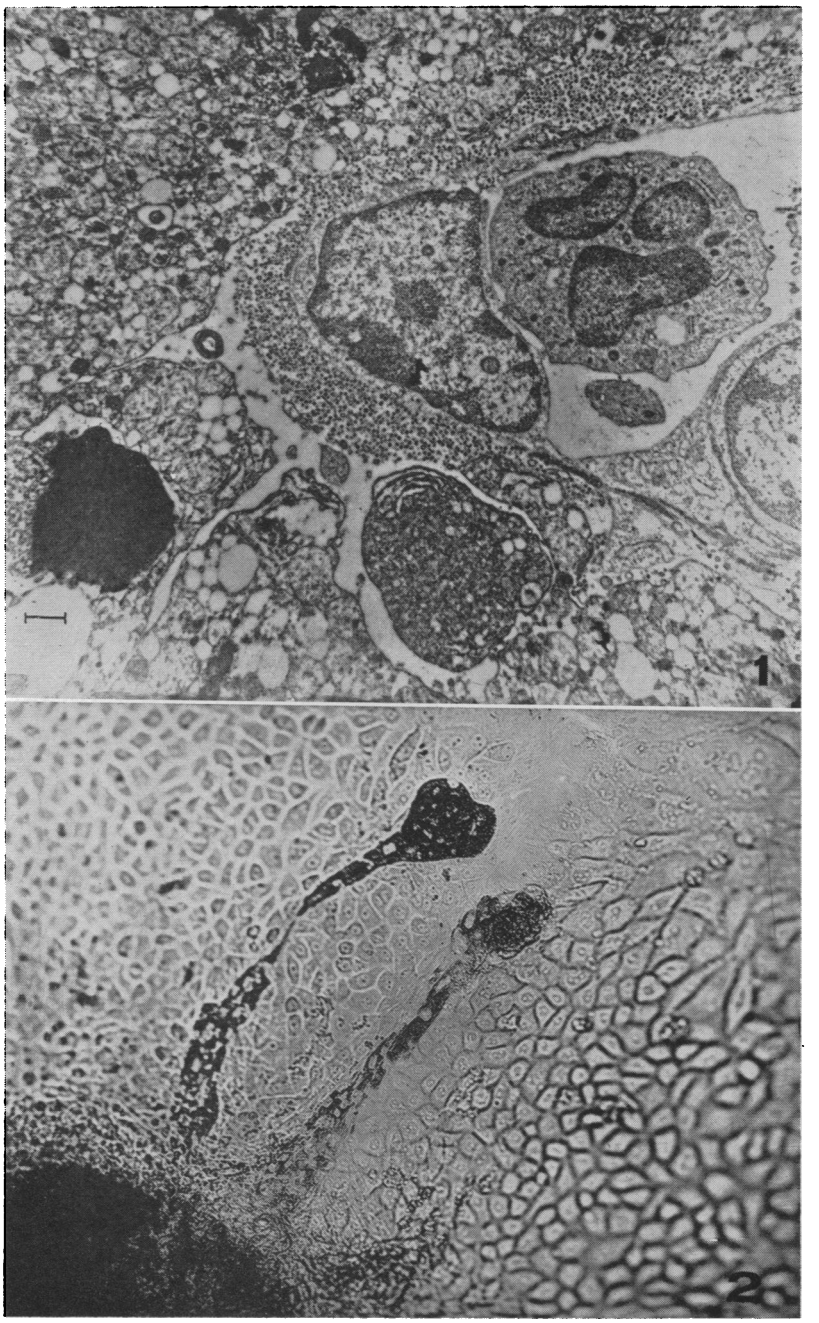

FIG. 1.—Electron micrograph of mouse liver infected with virus of mouse hepatitis. Virus may be seen accumulated along inner border of endothelium of capillary. (×7,000.)

FIG. 2.—Outgrowth of liver cords from newborn mouse liver. Clear epithelium surrounding these cords may have come from superficial epithelium of liver surface. Liver cell growth of this kind is occasionally obtained on glass. (See text. ×200.)

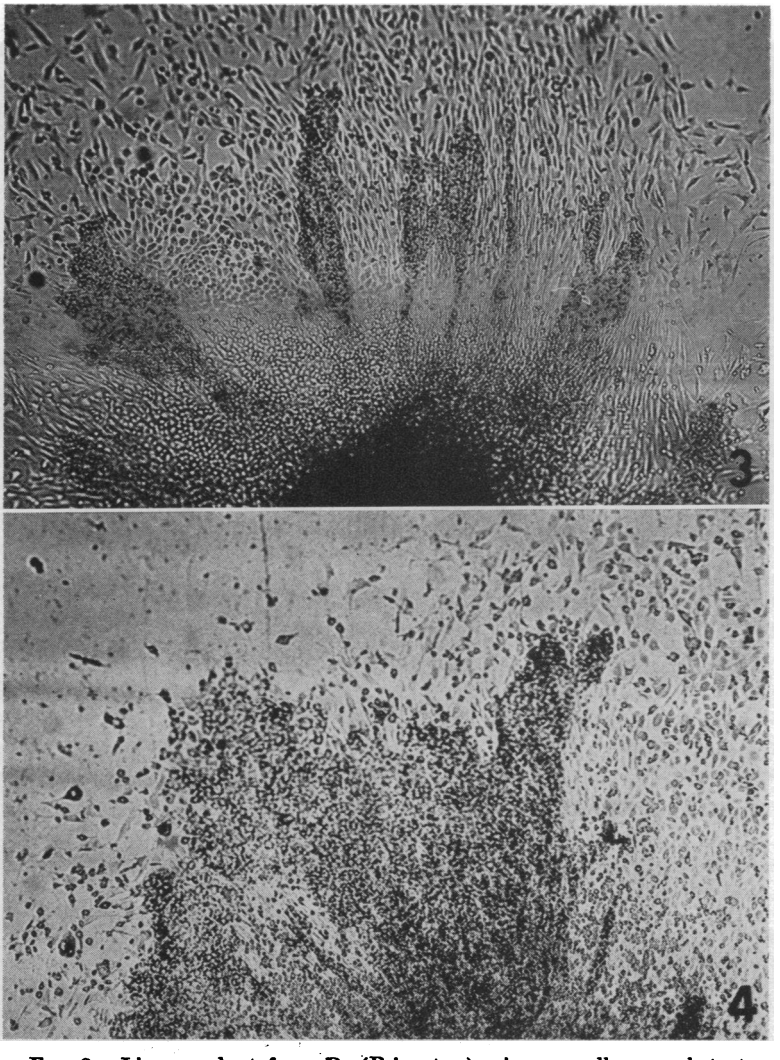

FIG. 3.—Liver explant from Pr (Princeton) mice on collagen substrate. Along with liver cell cord growth are masses of fibroblasts and macrophages. (About 150×.)

FIG. 4.—Outgrowth from liver of newborn C_3H mouse. Sheet of liver cells in middle. Macrophages wandering away from explant. (About 150×.)

characteristic shape and distribution, their migration away from the main explant, and their rapid uptake of neutral red (0.005 per cent). Macrophage degeneration began one to two days following inoculation of the virus and progressed to extensive destruction by four days (Figs. 5–8).

After observing the growth of macrophages in the horse serum cultures and their destruction, the amount of virus was determined at intervals in horse serum and chicken serum cultures. The only difference in appearance between the two media was the lack of migrating macrophages in the chicken serum cultures. The cultures in both media were inoculated three days following explantation with a

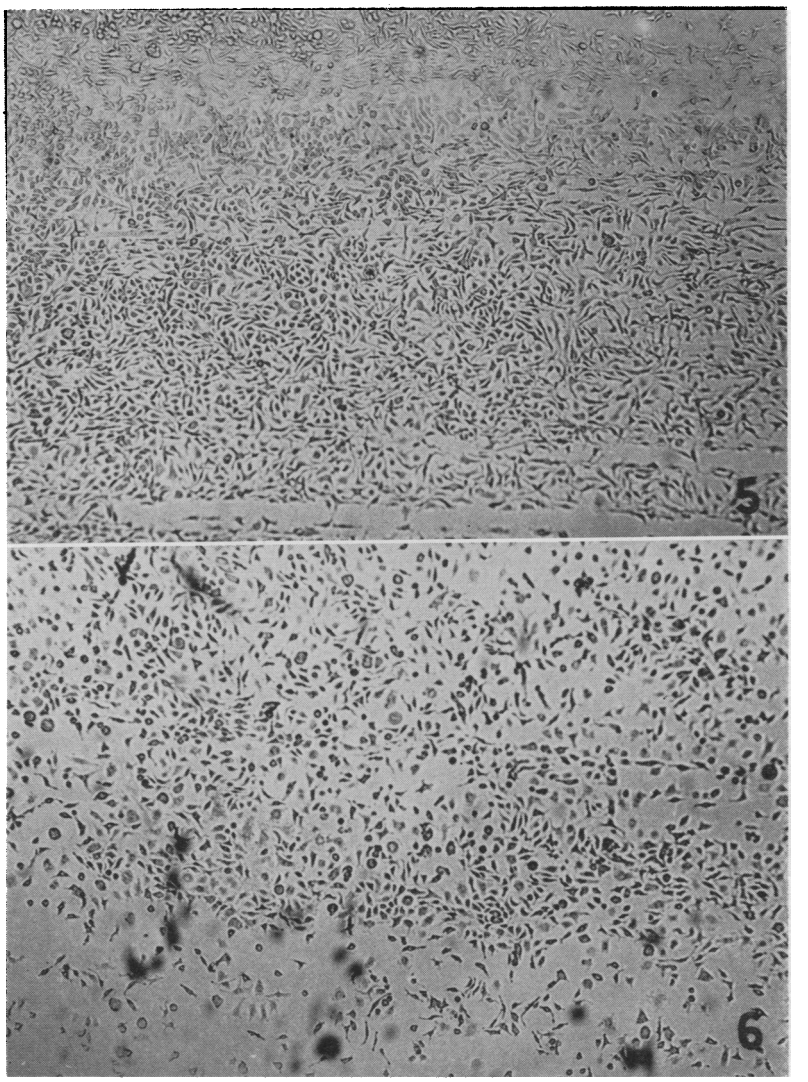

FIG. 5.—Mass of normal macrophages which have collected on glass at edge of collagen substrate. Pr mice. Part of the area is out of focus because of curvature of roller tube. 5-day culture. (About 150×.)

FIG. 6.—Originally similar to Fig. 5. One day of infection with mouse hepatitis. Scattered dark cells which have begun to round up. (About 150×.)

10^{-2} dilution of the stock liver suspension (titering $10^{-7.5}$). In the horse serum group, macrophage degeneration began two days following inoculation, and only scattered cells survived by the fourth day. The chicken serum group, however, which had no macrophages, showed no cellular change. The results of the titration of the supernatant fluids from both groups of cultures is shown in Figure 9. At six hrs there was a hundredfold difference, the horse serum being the higher. From 24 to 96 hrs there was no significant difference between the two; at six days the chicken serum was higher by about two log dilutions (two logs). It is possible

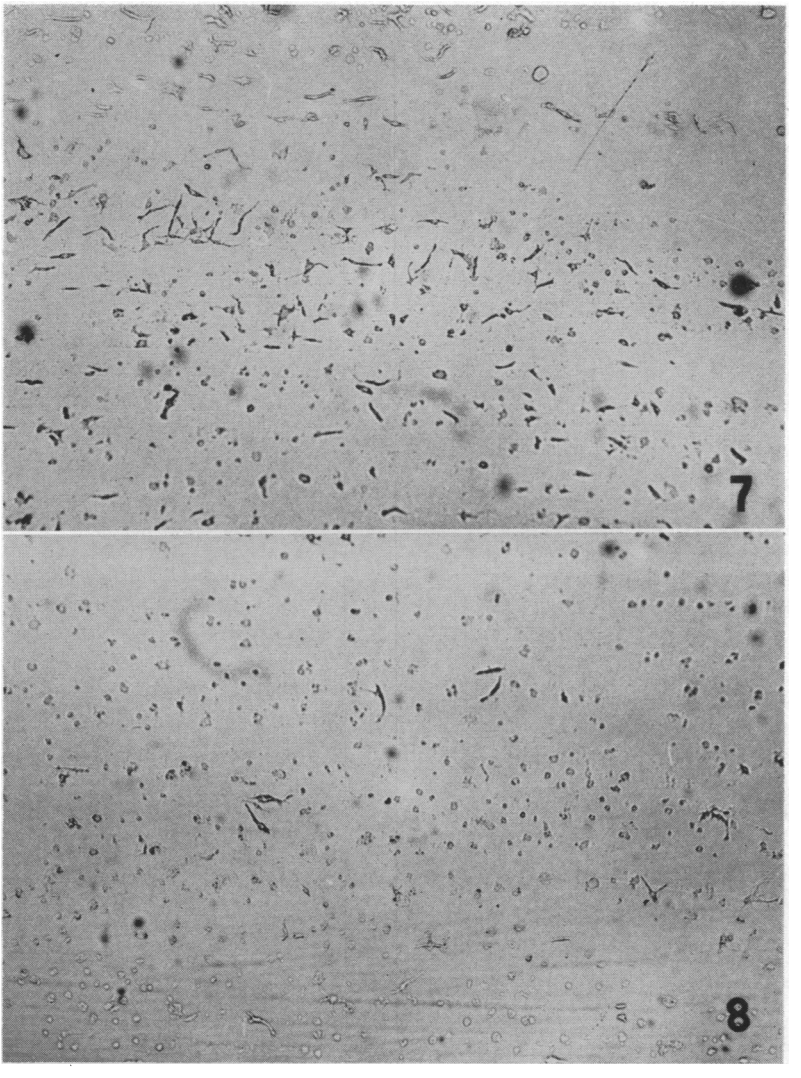

FIG. 7.—Originally similar to Fig. 5 but two days of infection. No normal cells left. Scattered partially damaged elongated cells. (About 150×.)
FIG. 8.—Originally similar to Fig. 5 but three days of infection. Remnants, or ghosts, of macrophages scattered throughout. Most destroyed cells have fallen off into the medium. (About 150×.)

that the reticulo-endothelial cells in the chicken serum fail to migrate but support virus growth.

A series of 11 tissue culture passages was made. Virus in amounts of 10^4 and 10^4 MID_{50} was recovered at each passage. Partial destruction of the liver parenchymal cells (which began about six days after inoculation of the virus and three to four days following macrophage destruction) appeared in the sixth tissue culture passage. There was no significant increase in the amount of tissue culture-adapted virus (Fig. 10).

In two experiments, macrophages from heart and lung tissue cultures were

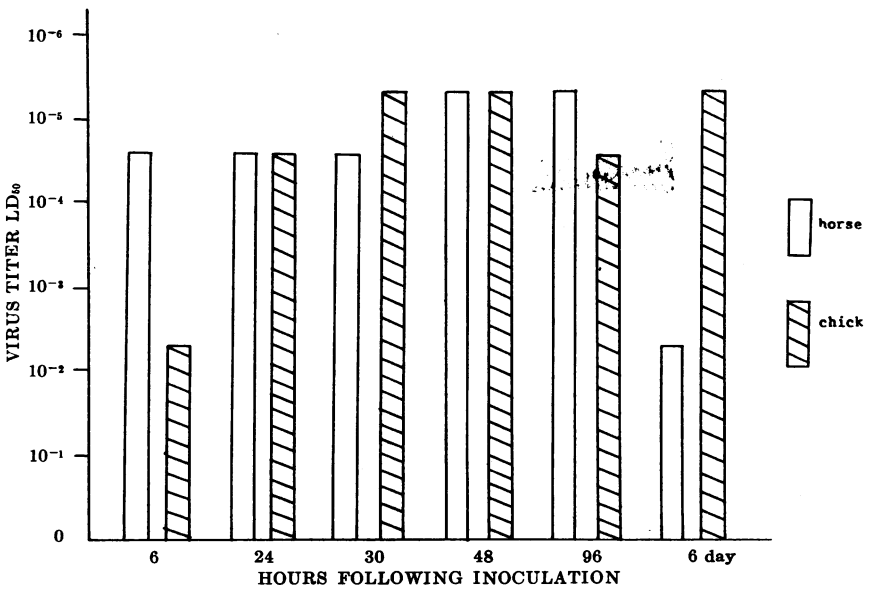

FIG. 9.—Growth curve of mouse hepatitis virus in tissue culture of mouse liver on collagen—horse serum vs. chicken serum.

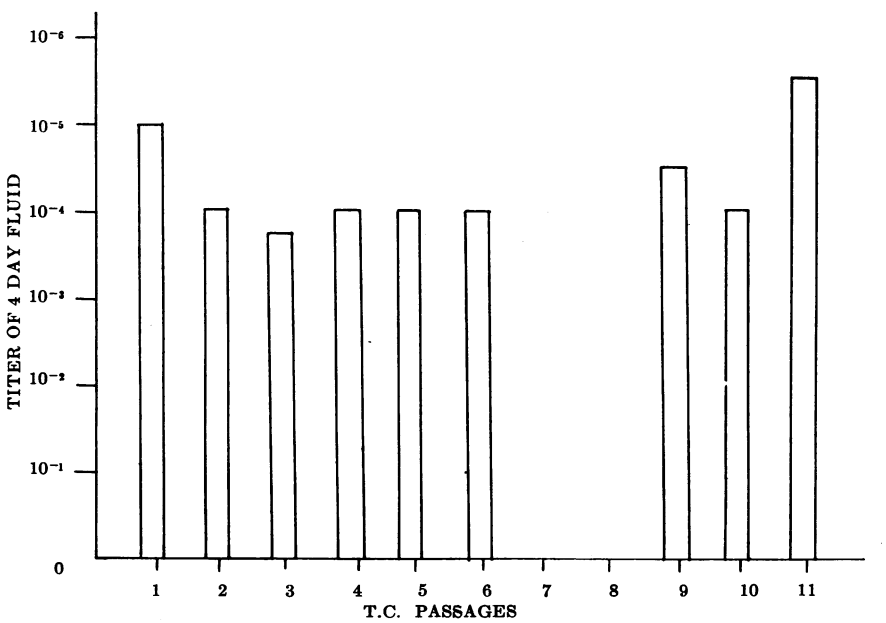

FIG. 10—Passage of mouse hepatitis virus in tissue culture of mouse liver cells on collagen—(horse serum medium).

destroyed by the virus, the other cell types remaining intact and 10^4 ID_{50} of virus was recovered from the supernatant of these cultures.

The amount of virus produced during the first 48 hr of infection was determined on the ninth tissue culture passage of virus. Macrophage and colony degeneration

began at two days, proceeded rapidly to extensive degeneration by three days, whereas only moderate degeneration of the colony itself was present by seven days. Titrations done at 18, 24, and 48 hr gave an average recovery of $10^{-3.7}$ ID_{50} of virus.

Genetic resistance: The C_3H (Andervont) strain of mice, free of mammary tumor agent and maintained as an inbred stock in our laboratory for 13 subsequent generations, was tested for susceptibility to mouse hepatitis virus and was found to be resistant to the virus, even when inoculated intraperitoneally with an undiluted suspension of the stock liver, which titered to $10^{-6.5}$ to $10^{-7.5}$. Tissue cultures of newborn mouse liver were prepared in the same manner as that previously described for the Pr mice. In the original colony outgrowths the liver parenchymal cells tended to grow in sheets rather than fingers, as compared to the Pr mice (Figs. 3 and 4). These liver cells remained intact throughout the experiment, whereas in the Pr cultures most of the liver cells disappeared after about eight days, in both control and experimental tubes. The macrophages migrated in the same manner in the two strains, spreading to the side of the tube opposite the colonies, but were fewer in number and slightly more granular in the C_3H.

In seven experiments, C_3H cultures were challenged with a 1:100 dilution of stock virus. The cultures were inoculated three to four days following explantation, by which time typical cell outgrowth had occurred. None of the inoculated cultures showed any macrophage destruction, nor destruction of any other cell type. Pr cultures, inoculated with virus at the same time, showed macrophage destruction within 48 hr.

In two experiments both C_3H and Pr cultures were inoculated with undiluted and 10^{-1} virus (ID_{50} $10^{6.5}$) to see whether heavy concentrations of the virus would affect the resistant cells. In the C_3H cultures inoculated with undiluted virus the macrophages became extremely granular, with some areas of definite degeneration. The macrophages did not, however, disintegrate (as the Pr macrophages always do following the usual infectious dose), but remained attached to the glass surface of the tube. When neutral red was added to the cultures seven days after inoculation, it was immediately taken up by most of the macrophages. Cultures inoculated with the 1:10 dilution showed scattered degeneration, and the higher dilutions (10^{-2} to 10^{-4}) remained healthy. The Pr cultures in these experiments showed initially the same peculiar effect of the undiluted virus, but subsequently degenerated, while the rest of the dilutions showed extensive destruction with only scattered surviving macrophages.

The question of humoral factors which may have persisted in the cultures and which were responsible for resistance or susceptibility was considered. All experiments were done after one change of medium. Specific tests on this point were done with two resistant strains kept in culture for seven and 11 days with two and four changes, respectively. These remained resistant. In four tests the Pr strain was found fully susceptible after 14 to 16 days in culture with four to five changes of medium. We have carried out two experiments combining the Pr and the C_3H liver explants in the same tube. Even though the macrophages were not clearly distinguishable from each other, about 50 per cent of the macrophages altogether were destroyed. It would seem therefore likely that the factors for resistance and susceptibility remain in the cells in culture.

Four other strains of mice have been tested for susceptibility to the virus, both

in vivo and *in vitro*. Preliminary results, with one or two experiments on each showed three strains to be susceptible: an obese mouse, CFW (Swiss), and the Webster (Swiss). The fourth, a lung tumor strain ("A" strain), was resistant. Complete agreement between mouse and macrophage susceptibility was obtained.

In order to determine the nature of this strain susceptibility, genetic crosses were made between the C_3H resistant and the Pr susceptible. The C_3H was obtained as an inbred strain and kept inbred for 13 additional generations, whereas the Pr strain has been inbred only since 1954 (about 20 generations). Tissue culture tests of four individual mice from the F_1 cross showed all cultures from all four mice to be susceptible; the macrophages were destroyed within two to three days. In the F_2 generation, five out of seven individual mice tested produced susceptible cells for a 71 per cent mouse susceptibility. For each individual mouse at least four cultures were prepared, two were used as uninoculated controls and two were inoculated with a 1:100 dilution of the stock virus.

Backcrosses were then begun between the F_1 and the C_3H (resistant). Tissue culture tests of the progeny of these mice resulted in three out of ten susceptible mice (30 per cent).

The comparable figures for the susceptibility of the weanling mice themselves, when the resistant strain is crossed with the susceptible, are given in Figures 11*a* and 11*b*. A single dominant factor for susceptibility would be expected to produce 100 per cent susceptibility in the F_1, 75 per cent susceptibility in the F_2, and 50 per cent in the backcross to the resistant strain. The data, although scanty, agree with this hypothesis.

Discussion.—Two facts, which may be related to one another, seem to have been established in these experiments. First, there is a specificity of cell type destroyed which may well be important in the pathogenesis of diseases affecting the reticuloendothelial system; and second, the apparent genetic basis of susceptibility of mice to this virus is reflected in the susceptibility of the macrophage in tissue cultures. The effects of viruses on chicken macrophages obtained from the buffy coat has been studied,[5, 6] and recently macrophages from peritoneal washings have also been followed in tissue cultures.[7] In most cases the cultures consisted almost entirely of macrophages, so that no direct comparison with other types of cells was possible. We have previously reported[8] briefly on the destruction of macrophages by mouse hepatitis when liver was grown on reconstituted collagen. Simultaneously Malmquist and Hay[9] described the selective destruction by the virus of African swine fever of macrophages in bone marrow cultures. Thus it would seem that a number of virus systems, perhaps those in which viremia plays a large role, may be diseases in which the reticuloendothelial system plays a significant role, and that present tissue culture techniques will uncover more situations where selective destruction of one cell type may be manifest. The large numbers of macrophages obtained in our cultures encourages us in the belief that mammalian macrophages from embryos may actually be grown in tissue culture.

It was then of special interest to find that the apparent genetic difference in susceptibility of mice was reflected in the resistance of the cells which were cultured from these mice. This statement is based on the fact that those two strains of mice found to be resistant as young weanlings also yielded resistant macrophage cultures from newborn individuals, whereas the four susceptible strains yielded

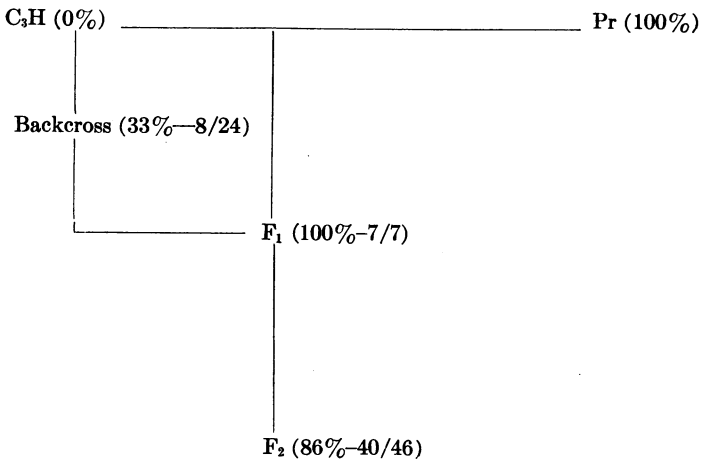

FIG. 11*a*.—Tests for mouse susceptibility.

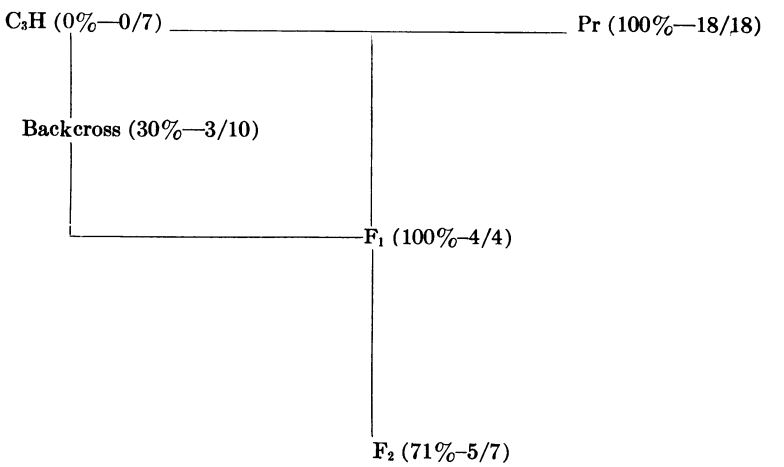

FIG. 11*b*.—Tests for macrophage susceptibility from individual mice.

susceptible cells. Furthermore, the genetic hybrids between the resistant and susceptible strains yielded susceptible cells, and the backcross to the resistant strain and the F_2 generation yielded evidence of segregation of resistant and susceptible progeny. The factor for susceptibility may be concluded to be dominant, and possibly unifactorial, but further studies are necessary to establish this. A more complete study of inheritance of disease susceptibility was reported by Sabin, but no tissue culture comparison was made.[10] Within the limits of the number tested, the character for susceptibility segregated in similar fashion both in the weanling mice and in the tissue culture experiments. Final proof of the relationship of this susceptibility in tissue culture would presumably rest on the culturing of cells from individual mice and subsequent determination of their individual susceptibility.

In all of our experiments we have used a virulent strain of mouse hepatitis virus. The relationship of our findings to the established role of the protozoan *Epierythro-*

zoon coccoides has not been studied. The possibility that our tissue cultures from resistant mice are resistant because of latent infection in the mice themselves seems quite remote. The factors should not, in this case, segregate in a Mendelian fashion, and latent infection of young or newborn mice has not been reported in these infections.

Summary.—A virulent strain of mouse hepatitis virus is shown to have a selective destructive effect on the macrophages cultured from the liver and other tissues of newborn mice, and no apparent effect on the fibroblasts and epithelial cells. Tissue susceptibility seems therefore to be a property of the reticulo-endothelial system. Cultures obtained from resistant strains of mice showed no destruction of macrophages, whereas susceptible strains of mice yielded macrophages which were destroyed in culture. Tests of hybrids resulting from crosses between resistant and susceptible strains indicate that susceptibility is inherited and that genetic segregation of susceptibility and resistance occurs in the F_2 and backcross generations. This is apparent both in the mice themselves and in cultures obtained from the different genetic crosses.

* This investigation was conducted under the auspices of the Commission on Viral Infections of the Armed Forces Epidemiological Board, and was supported (in part) by the Office of The Surgeon General, Department of the Army.

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*FREQUENCY OF DELETIONS AMONG SPONTANEOUS AND INDUCED MUTATIONS IN SALMONELLA**

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Communicated June 21, 1960

Among mutants collected at random in microorganisms, two classes are distinguishable: those that have the capacity to revert to the wild type, and those that cannot revert. Mutants of the first class are demonstrably the result of mutation at a single site of a gene locus, whereas the nonreverting mutants are due to mutation affecting two or more adjacent sites. These so-called multisite mutants, since they show properties characteristic of the deletion mutants found in higher organisms, are assumed to originate by deletion of a small segment of the gene string.

As a rule the frequency of deletions is low as compared with the frequency of single-site mutations. In *Salmonella typhimurium* strain LT-2, for example, only 20 (4 per cent) deletions have been found among 495 spontaneous mutations affect-