Interaction of Mouse Peritoneal Macrophages with Fixed Rabies Virus in vivo and in vitro

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SUMMARY

The resistance of mice to intraperitoneal and intramuscular infection with fixed rabies virus increases with age. Treatment of mature animals with either silica, Indian ink or antimacrophage serum, which are cytotoxic for macrophages, reduced their resistance to intraperitoneal, but not to intramuscular or intracerebral infection. Transfer of peritoneal macrophages from adults to syngeneic suckling mice delayed but did not prevent mortality from intraperitoneal infection: transfer of peritoneal macrophages to intramuscular sites of infection did not protect adult mice.

Rabies virus was phagocytosed by peritoneal macrophages in culture but neither replicated nor induced interferon. Evidence of active intracellular destruction of virus was obscured by thermal inactivation at 37 °C. Less inactivation occurred at 33 °C. Infected macrophages from suckling mice, but not those from adult mice, spread infection to susceptible cells.

INTRODUCTION

Heritable resistance factors, interferon induction, metabolic differences in macrophages from different sites and functional differences between stimulated or unstimulated macrophages from immature or adult hosts have all been shown to influence macrophage activity against viruses. (Goodman & Koprowski, 1962; Smith & Wagner, 1967; Hirsch, Zisman & Allison, 1970; Tompkins, Zarling & Rawls, 1970; Stevens & Cook, 1971). Numerous observations in different host–virus systems have shown that macrophages can inhibit, enhance or perhaps be irrelevant to the spread of infection (Mims, 1964; Johnson, 1964; Gresser & Lang, 1966; Silverstein, 1970; Zisman, Hirsch & Allison, 1970). Apart from the probability that most viruses are taken up by macrophages, it is not yet possible to generalize about their subsequent fate or about the influence of macrophages on the outcome of virus diseases; and it appears that the importance of virus-macrophage interaction requires individual assessment with each virus.

The resistance of mice to extraneural inoculation with several neurotropic viruses increases with age, and resistance to herpes encephalitis is related to macrophage maturity (Johnson, 1964; Nelson, 1969; Zisman et al. 1970; Stevens & Cook, 1971). Age-dependent resistance of mice to extraneural inoculation with both street and fixed rabies virus has also been demonstrated (Casals, 1940) but its relationship to macrophage activity is unclear. The interaction of 2 fixed strains of rabies virus with mouse peritoneal macrophages was examined using the experimental designs developed for herpes virus by Zisman and co-workers (1970); and by Hirsch and his colleagues (1970).
METHODS

Viruses. Two strains of rabies virus were used. Stocks of CVS virus were prepared from 10% suspensions of infected mouse brain. Flury LEP virus—adapted to growth in BHK21 cells—was obtained as infected culture fluid from Miss J. Crick, Animal Virus Research Institute, Pirbright, Surrey. Both viruses were stored at $-170\, ^\circ\text{C}$.

Virus assays. Samples were tested for infectivity by intracerebral titration in mice. Adult mice (11 to 14 g) were used for titrating CVS; Flury virus was titrated in suckling mice (5 to 7 days old). At least 5 mice were inoculated with each dilution. Median lethal doses were calculated by the methods of Reed and Muench or Spearman–Kärber (Lorenz & Bogel, 1973), virus concentrations were expressed as intracerebral (IC)LD$_{50}$/ml.

Mice. Random-bred T.O. or C.S.I. mice were used for virus titrations, as a source of peritoneal macrophages, and for testing age-susceptibility to peritoneal or intramuscular infection with rabies virus. Inbred CBA mice were used for macrophage transfers.

Treatment of mice with Indian ink and silica. Twenty-four hours before infection with rabies virus, adult mice were inoculated intraperitoneally (i.p.) either with 1.0 ml of Pelikan ink (10%, v/v, in saline), or with 1.0 ml of saline containing quartz dust (50 mg/ml). The latter was kindly donated by Dawson & Dobson Ltd, London, and prepared and sterilized as described by Zisman et al. (1970).

Treatment of mice with antimacrophage serum (AMS). Adult mice were inoculated i.p. with 3 x 0.3 ml doses of AMS, given 24 h before, at the same time as, and 24 h after infection with rabies virus. Rabbit antimouse macrophage serum was prepared and tested by the methods of Hirsch, Gary & Murphy (1969). Its antimacrophage activity was also assessed radioactively from the reduction of phagocytosis of Escherichia coli labelled with $[^3\text{H}]$-leucine, after the method of Schroit, Geiger & Gallily (1973).

Preparation and culture of macrophages. Mice received no previous stimulation. Cells were obtained from either adult or suckling mice (3 to 5 days old) after irrigating their peritoneal cavities with Eagle's minimal essential medium (MEM) containing heparin, 5 units/ml. Leighton tubes were seeded with $10^6$ to $10^7$ cells suspended in 1.0 ml volumes of MEM + 40% horse serum. Cultures were incubated at 37°C in an atmosphere of 5% CO$_2$. Non-adherent cells were removed by vigorous washing after 2 and 4 h incubation. Culture medium was replaced after each wash and again after 24 h incubation, when between 1 and $5 \times 10^6$ adherent cells per tube were obtained. More than 90% had the morphology and phagocytic properties of macrophages and were regarded as normal or ‘unstimulated’ macrophages.

Inoculation and sampling of macrophage cultures. The fluid from each culture was replaced with fresh medium (0.5 ml) containing rabies virus ($10^6$ IC/LD$_{50}$) and incubated either at 37 °C or 33 °C. Cultures were sampled at different times and at each interval 4 cultures were removed and cooled to 0 °C. One tube was set aside for fluorescent antibody staining and the three remaining tubes were washed once with phosphate buffered saline (PBS), once with antirabies serum and a further four times with PBS. Cells were then detached into fresh medium by shaking with glass beads, and disrupted by ultrasonic vibration. Samples of the final washing fluid and the pooled cell lysates were stored at $-170\, ^\circ\text{C}$ until they were titrated for virus.

Spread of infection from macrophages. Suspensions containing $2 \times 10^6$ peritoneal cells/ml were obtained from either adult or suckling mice. Two drops of either suspension were seeded on sterile microscope slides at approx. 1 cm from the edge. The slides were incubated individually in Petri dishes at 37 °C for 24 h. Non-adherent cells were removed by vigorous
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washing. The macrophages from each drop populated approx. 9 mm² representing 1% of the total area of the slide. They were infected with 0.02 ml of Flury virus containing 10^6 ICDL₀₀/ml. After 4 h adsorption at 37 °C, the cells were washed as described to remove free virus and overlaid with 20 ml of a suspension containing 4 x 10^6 BHK cells. A confluent growth of BHK cells covered the entire slide after 18 h incubation at 37 °C; the growth medium was then replaced with maintenance medium and the slides were incubated for 72 h at 33 °C in 5% CO₂. Uninfected slide cultures of both types of macrophages and BHK cells were prepared, as well as infected and uninfected control cultures of BHK cells alone. After incubation all slides were fixed in cold acetone and stained with fluorescent antibody.

Flury virus and BHK 21 cells. Clone 13 cells were grown in BHK medium (Wellcome)+ 10% calf serum. They were infected at the same time and multiplicity as mouse macrophages and similar adsorption, washing and sampling procedures were applied. They were incubated either at 33 °C or 37 °C.

Fluorescent antibody staining. Both indirect and direct methods were used; for the former, hyperimmune rabbit antirabies serum was followed by swine antirabbit conjugate (Nordic-Pharmaceuticals, London). Fluorescein labelled goat antirabies globulin was prepared by the method of Schneider (1973) for direct staining. Smears of either normal mouse brain or mouse brain infected with CVS virus were used as negative and positive control material. Controls of normal and antirabies serum were included in the staining procedures which were those of Sowa, Collier & Sowa (1971).

Interferon assays. Interferon was estimated by vaccinia plaque reduction in mouse embryo fibroblast cultures (Gifford, 1963). A reference preparation containing a nominal 1000 international units of interferon was included in all titrations.

RESULTS

Age-dependent resistance

Results similar to those reported by Casals (1940) were obtained when CVS virus was titrated by i.p. or intramuscular (i.m.) inoculation in mice of varying ages. Resistance to infection by both routes of inoculation was highly dependent on age but was most marked in those inoculated intraperitoneally (Fig. 1).

Effect of Indian ink, silicia and antimacrophage serum (AMS)

The resistance of adult mice to i.p. infection was significantly reduced if Indian ink, silica or AMS were inoculated by the same route at suitable times before or during infection (Fig. 2). The results with Indian ink confirm those of Marie (1932) and together with the effects of silica and AMS, which are specifically cytotoxic for macrophages, suggest that age-dependent resistance to fixed rabies virus is related to macrophage maturity.

Although macrophages may prevent the spread of rabies infection from the peritoneal cavity to the central nervous system their role in preventing infection from other sites is uncertain. Neither silica nor AMS increased the susceptibility of adult mice to either i.m. or i.c. infection with fixed virus, although both these agents are reported to impair the function of macrophages at sites remote from their point of application (Zisman et al. 1970).

Macrophage transfer

The resistance of adult mice to i.m. inoculation with rabies virus was not increased by the transfer of 10^6 peritoneal macrophages to the site immediately before infection. Transfer of 10^6 to 10^7 adult macrophages to the peritoneal cavity of groups of 10 or more syngeneic
Fig. 1. Development of resistance with age to fixed rabies virus. CVS was titrated either intra-peritoneally (●-●) or intramuscularly (○-○); 5 to 10 mice/dilution. Median lethal doses for each route were calculated in terms of intracerebral LD₅₀ for 21 day old mice.

Fig. 2. The effect of various treatments on the resistance of adult mice to i.p. infection with 10⁷ ICLD₅₀ CVS. ●-●, 50 mg silica 24 h before infection; ▲-▲, 1 ml 10% Indian ink 24 h before infection; ■-■, 0.3 ml antimacrophage serum 24 h before, with and 24 h after infection; ○-○, virus only; ○ △ □, silica, Indian ink or antimacrophage serum only.
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suckling mice delayed the average time of death by 1 to 2 days, but did not reduce mortality after i.p. infection with as little as 10 LD₅₀ of fixed rabies virus. Hirsch et al. (1970) also reported similar results with herpes virus and unstimulated macrophages. These observations suggest that macrophages from mice of different ages may vary in their ability to inactivate rabies virus, release ingested virus particles or permit viral replication. The fate of rabies virus was therefore examined in isolated macrophages cultured in vitro since resistance to infection in vivo need not be solely associated with macrophage activity (Nelson, 1969).

**Phagocytosis of rabies virus by macrophages**

Virus titrations on macrophages taken from cultures at different times after infection indicated that the uptake of CVS virus adult macrophages was maximal between 2 and 3 h (Fig. 3). The maximum amount of virus recovered, however, represented only 10 to 20% of that inoculated. This probably represents only virus within the cells since surface associated virus would be neutralized by the sampling procedure (see Methods). Poor recoveries of herpes virus from macrophages have also been reported (Johnson, 1964). No virus was found in samples of the final washings before cells were disrupted and only traces were detected in macrophages infected and held at 0 °C suggesting that rabies virus was actively phagocytosed.

**Infectivity of rabies virus in macrophages**

Neither CVS nor Flury virus grew in cultures of adult mouse macrophages incubated at 37 °C. Both strains were inactivated biphasically; the rates were similar to those found when virus in the same concentration as that found within macrophages was incubated in cell-free media (Fig. 4). Inactivation could not therefore be attributed solely to degradation by cellular activity within macrophages.
Fig. 4. Inactivation of CVS and Flury virus in cultures of mouse macrophages and in cell-free medium at 37 °C. Intracellular virus: ●, Flury; ▲, CVS. Virus in cell-free medium: ○, Flury; △, CVS.

Fig. 5. Infectivity of Flury virus at 33 °C in BHK cells (● --- ●), in adult mouse macrophage cultures (□ --- □), in suckling mouse macrophages (△ --- △), or in cell-free medium (○ --- ○).
Adapted samples of Flury virus grow optimally in BHK cell cultures incubated at 33 °C. The thermal inactivation rate of the virus in cell-free medium was less at this temperature than at 37 °C. However, it is not clear whether significant growth or inactivation occurred in macrophages incubated at 33 °C; certainly inactivation in suckling macrophages was approx. one tenth that in the adult cells, and limited growth of virus may have masked inactivation in the former cells. Nevertheless, during the same observation period, virus titres in similarly infected BHK cells increased approx. 1000-fold (Fig. 5).

No obvious cytopathic changes occurred in either adult or suckling mouse macrophages observed for 4 to 5 days after infection with rabies virus. Samples of all the cultures were examined after staining with fluorescent antibody (see Methods). Although specific fluorescent material was seen in infected but not uninfected macrophages it appeared irregularly during the observation period and neither the amount nor its distribution permitted any assessment of the fate of the virus by this method. By contrast, many BHK cells showed fluorescent foci 24 h after infection. Fluorescence increased in subsequent samples until at 96 h almost all cells showed typical cytoplasmic granules or aggregates of positively stained material (Wiktor, 1973).

**Interferon production**

Interferon is produced by macrophages (Smith & Wagner, 1967; Hirsch *et al.* 1970) and the restriction of the growth of rabies virus even in susceptible cells has been attributed to interferon induced by infection (Wiktor & Clark, 1972). However, no interferon was detected in uninfected macrophage cultures or in those infected with either CVS or Flury virus after 24 and 48 h incubation at either 37 or 33 °C. The failure of rabies virus to grow in mouse macrophages therefore appears to be unrelated to interferon induction.

**Spread of infection**

There were pronounced differences between the ability of adult and of suckling mouse macrophages to disseminate infection. BHK cells co-cultivated with infected adult macrophages showed only scanty fluorescent foci in cells close to the area of the original macrophage explants (see Methods). However, almost the entire monolayer of BHK cells showed rabies-specific fluorescence when stained after cultivation with infected suckling mouse macrophages, indicating that macrophages from sucklings spread rabies infection more readily than those of adults.

**DISCUSSION**

Reduction of the resistance of adult mice to intraperitoneal infection with rabies virus by agents cytotoxic for macrophages suggests that these cells behave toward rabies virus in a manner analogous to that reported for herpes virus (Zisman *et al.* 1970). Kliger & Bernkopf (1943) traced the fate of an unspecified strain of rabies virus in mice after intraperitoneal inoculation and found that 6 h after infection by this ‘abnormal’ route, virus was absent from the peritoneal cavity and local lymph glands. Virus reappeared 4 days later, in that segment of the spinal cord related to the peritoneal region, in mice 3 weeks old but not in those 3 months old – of which more than 40% survived. These results are explicable in terms of our experiments on the uptake of virus and its dissemination by macrophages of young but not mature mice. It is also interesting to speculate on the possible involvement of macrophages in non-fatal or abortive rabies which can be more readily induced in mice inoculated intraperitoneally than it can by other routes (Bell, 1966).

Infection with rabies virus ‘normally’ occurs after subcutaneous or intramuscular entry, and persistence of rabies virus at the inoculation site has frequently been reported. Recent
observations suggest that some street strains of rabies virus multiply initially in muscle cells and rabies-infected macrophages have been identified in bone marrow late in these infections (Murphy & Bauer, 1974). However, our results indicate little macrophage activity against intramuscular infection, although they suggest that if macrophages monitor these areas, sequestration of rabies virus within them is possible.

With several viral encephalitides, spread to the central nervous system by neural, olfactory or haematogenous routes occurs after initial growth in non-neural cells at the site of virus entry. These entry sites may be monitored by macrophages which in some instances also support viral multiplication (Johnson & Mims, 1968; Silverstein, 1970). Although our macrophage cultures did not support the growth of infectious rabies virus even with a strain well adapted to growth in non-neural cells, it is possible that defective (non-infectious) particles replicated. However, Johnson (1964) briefly noted that rabies virus grew neither in adult nor suckling mouse macrophages and T. J. Wiktor (personal communication) has obtained similar results. The failure of rabies virus to grow in macrophages was not attributable to the induction of interferon which is known to take place in these cells (Smith & Wagner, 1967; Hirsch et al. 1970). In pronounced contrast to these observations, the type strain of the rhabdovirus group, vesicular stomatitis virus, grows readily in macrophages from mice and other species (Eustatia et al. 1972).

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REFERENCES


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