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## Replication of Human Respiratory Coronavirus Strain 229E in Human Macrophages

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### SUMMARY

Evidence for the replication of human coronavirus strain 229E (HCV 229E) in macrophages is presented. Virus antigen was detected in macrophages by an immunofluorescent technique 24 h after infection and virus particles were observed in the cisternae of the endoplasmic reticulum by electron microscopy. Giant cells were observed by light and scanning electron microscopy, and large multinucleate cells were seen by thin-section electron microscopy, suggesting that HCV 229E can induce syncytial formation in cultured human macrophages. Furthermore, the production of infectious virus by macrophages was demonstrated by an infectious centre assay.

### INTRODUCTION

The cells of the reticuloendothelial system play a key role in determining susceptibility to virus infections (Mims, 1964; Silverstein, 1970; Morgensen, 1979). Studies with a number of different viruses have shown a correlation between the growth of virus in macrophages and the susceptibility of the host to infection (Bang & Warwick, 1960; Roberts, 1963, 1964; Tosolini & Mims, 1971; Virelizier & Allison, 1976). Coronavirus infections of the respiratory tract are widespread in the human population and account for a significant proportion of colds (McIntosh, 1974; Monto, 1974). However, there are few data available concerning the interactions between human respiratory coronaviruses and macrophages. In view of the importance of macrophages in virus immunity, the interactions between the human respiratory coronavirus strain 229E (HCV 229E) and human macrophages have been investigated. The results of immunofluorescence, electron microscopic and infectivity studies on human macrophages infected *in vitro* show that these cells can support the replication of HCV 229E.

### METHODS

**Virus.** HCV 229E was grown in monolayer cultures of diploid cells of the MRC continuous (MRCc) line as described previously (Macnaughton & Madge, 1978). The cell monolayers were infected at an input multiplicity of 0.1 infectious units/cell and, after an adsorption period of 1 h at 33 °C, were incubated at 33 °C for 32 h in Eagle's basal medium with 2% newborn calf serum. Virus was clarified by centrifugation at 2000 g for 30 min at 4 °C and stored at -70 °C prior to use.

**Macrophages.** Human peritoneal macrophages were obtained from renal patients undergoing peritoneal dialysis (Fakhri *et al.*, 1978; Ganguly *et al.*, 1980). Cells from overnight dialysis washings were concentrated by low-speed centrifugation and resuspended in RPMI 1640 bicarbonate medium with 10% foetal calf serum, penicillin and streptomycin. Cells

were adsorbed on to 10 mm diam. coverslips in 5 cm Petri dishes,  $3 \times 10^6$  cells per dish, for 40 min at 37 °C, washed four times with medium and then overlaid with 5 ml medium. Fibroblasts were always present in cultures and represented approx. 2% of the total cell population. After 48 h in culture over 90% of control uninfected cells were viable by the trypan blue exclusion test. Cultures were infected after 24 h with 0.5 ml HCV 229E at an input multiplicity of 2 infectious units/macrophage and then cultured for 24 h at 33 or 37 °C. In some experiments heat-inactivated virus (1 h at 56 °C) was used. At 24 h post-infection cells were processed for immunofluorescence, scanning and thin-section electron microscopy.

*Preparation of antisera.* Immune serum against HCV 229E was prepared in New Zealand white rabbits as described previously (Kraaijeveld *et al.*, 1980). A 0.5 ml portion of purified virus was mixed with an equal volume of Freund's complete adjuvant and then injected intracutaneously at up to 20 different places in the shaven back of a rabbit. Two days before immunization, animals received 0.5 ml *Bordetella pertussis* vaccine (Lister Institute of Preventive Medicine, Elstree, Middx, U.K.) intracutaneously as an additional adjuvant. Animals were bled before and 6 weeks after immunization and the sera stored at -20 °C. Before use, sera were adsorbed with MRCc cells at 4 °C for 16 h.

*Indirect immunofluorescence.* Infected and uninfected macrophages on 10 mm diam. coverslips were fixed with methanol and processed for indirect immunofluorescence using immune serum to HCV 229E as described previously (Macnaughton *et al.*, 1980). Fluorescence was observed under oil-immersion with a Nikon SKE microscope fitted with a Projectina epi-fluorescence attachment.

*Infectivity assay.* Macrophage cultures were infected with virus at 33 °C as described above, and after 2 h, were washed twice with medium and incubated a further 22 h at 33 °C. Aliquots were taken after washing and 24 h after infection and tested for infectious HCV 229E particles by an infectivity focus assay (Macnaughton *et al.*, 1980).

*Infectious centre assay.* Cells were adsorbed to 10 mm diam. coverslips in 5 cm plastic Petri dishes for 40 min at 37 °C as described above. Cultures were then treated with a mixture of 0.25% trypsin and 0.1% versene for 5 min at 37 °C and subsequently washed four times with medium. Adherent macrophages are resistant to detachment by trypsin (Dr R. Dean, personal communication) and, hence, the trypsin treatment step was introduced to reduce the number of contaminating fibroblasts. Cultures were incubated for 24 h at 37 °C, and then coverslips with their adherent cells transferred to 3 cm diam. Petri dishes and infected with virus at 33 °C. Uninfected cells were taken for scanning electron microscopy at this stage so that the total number of fibroblasts per coverslip could be counted and the number of macrophages estimated. At 1 h post-infection cells were washed twice with medium and then incubated for 1 h at 33 °C with 200  $\mu$ l of immune rabbit serum to HCV 229E (neutralization titre of 1 in 1024 by tube titration using  $10^3$  p.f.u. of virus) to neutralize unadsorbed virus. After a further two washes with medium approx.  $10^6$  MRCc cells were added to each Petri dish and incubated for 6 h at 33 °C. The cells were then covered with an overlay medium containing 1% sodium carboxymethylcellulose and 0.08% tyrosine and incubated for a further 40 h at 33 °C. The overlay medium was removed, the coverslips fixed in methanol and processed for indirect immunofluorescence. The number of fluorescent foci on the coverslips was determined as described previously (Macnaughton *et al.*, 1980). Coverslips without macrophages were treated with virus and then processed as described above.

*Scanning electron microscopy.* Macrophages on coverslips were fixed for 24 h at 4 °C with 3% glutaraldehyde in 0.1 M-cacodylate buffer pH 7.3 containing 5% (w/v) sucrose. After washing with 0.1 M-cacodylate buffer, they were post-fixed for 1 h on ice in 1% osmium tetroxide buffered with 0.1 M-cacodylate buffer pH 7.3. They were then treated for

30 min with 2% tannic acid in 0.1 M-cacodylate buffer followed by 10 min in 1% osmium tetroxide. The cells were dehydrated through a graded series of ethanol (25 to 100% in water) and subsequently dried at the critical point of liquid CO<sub>2</sub> with a Polaron critical point drying apparatus (Polaron Equipment, Watford, Herts, U.K.). Dried coverslips were mounted on aluminium stubs using Electrodag 915 (Acheson Colloids, Plymouth, Devon, U.K.). A thin layer of gold was evaporated on to the cells in a Polaron E5100 sputtering apparatus before examination in a Philips PSEM 500 scanning electron microscope.

*Thin-section electron microscopy.* Macrophages were fixed for 1 h at 20 °C in 3% glutaraldehyde buffered with 0.1 M-cacodylate buffer containing 5% (w/v) sucrose. They were then fixed in 1% osmium tetroxide buffered with 0.1 M-cacodylate buffer pH 7.2 and, following washing in distilled water, stained with a 1% aqueous solution of uranyl acetate for 3 h at 20 °C. The cells were then gently scraped from the surface of the Petri dishes and pelleted in a warm (50 °C) 2% solution of low-melting point agar. After dehydration in acetone and propylene oxide they were embedded in Spurr's resin. Ultrathin sections were cut and stained for 1 min in Reynold's lead citrate. They were coated with carbon and examined in a Philips EM 300 electron microscope.

## RESULTS

Peritoneal macrophages from eight patients were used, six of which showed evidence of virus replication. In a typical experiment in which there was replication, 4% of infected macrophages appeared as giant cells, whereas no giant cells were seen in the uninfected culture after 24 h. In addition, the number of attached cells in the infected culture was reduced by approx. 50%, whilst in the uninfected culture there was a reduction of only 6% in the number of these cells. Approximately 70% of the attached infected cells were viable. About half of the attached cells in infected cultures fluoresced brightly on immunofluorescent staining with immune serum to HCV 229E (Fig. 1). Similar fluorescent cells were not observed in the uninfected control cultures.

A scanning electron micrograph of uninfected human macrophages is shown in Fig. 2. The majority of uninfected cells measured about 11 µm in their shortest axis and approx. 15 µm in their longest axis. The main cell body showed extensive ruffling, whilst at the periphery a thin, relatively smooth lamella extended out over the substratum. Single cells with a similar morphology were observed in virus-infected macrophage cultures. However, giant cells were also seen in infected cultures and these cells measured 25 to 30 µm in their shortest axis and up to 40 µm in their longest axis (Fig. 3). Except for their size, the morphology of these cells was similar to that of control macrophages. Occasionally, large cells were observed in control cultures. However, the morphology of these cells was quite different from the giant cells described above. These cells had an unruffled surface covered with small blebs and thin slender spikes extended over the substratum. Such cells are probably swollen, dead or dying macrophages.

Large multinucleate cells were observed by thin-section electron microscopy in infected cultures (Fig. 4). These cells accounted for about 10% of the total population. Similar multinucleate cells were not observed in control cultures. Mature virus particles were seen in the endoplasmic reticulum in approx. 15% of the cells examined. They had a mean diam. of 80 nm and consisted of a 50 nm core separated from an outer envelope by a space approx. 8 nm wide (Fig. 5a). Both single and multinucleate giant cells contained virus particles. These particles were considered to be newly synthesized virions rather than particles from the input inoculum since the titre of virus in the infecting inoculum was too low to allow visualization by electron microscopy, and virus was not observed in macrophages incubated with heat-inactivated virus. In addition, particles were observed budding into the swollen cisternae of the endoplasmic reticulum (Fig. 5b).

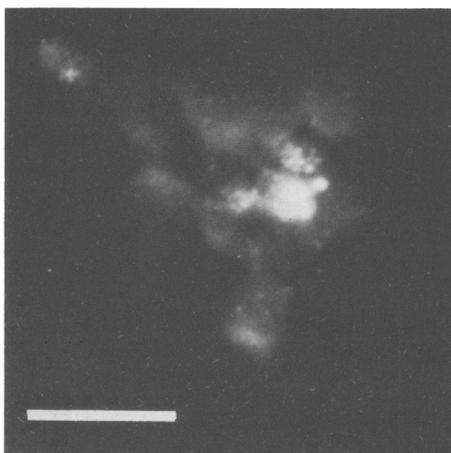


Fig. 1. Immunofluorescent staining of virus antigen in human macrophages 24 h after virus infection. Bar marker represents 20  $\mu\text{m}$ .

Macrophages from two patients were infected at 33 °C with HCV 229E and the number of infectious virus particles in supernatants taken after 24 h was determined by infectivity focus assay. Titres of about  $10^5$  infectious units/ml were obtained. Infectious virus was not detected in supernatants taken immediately after the 2 h adsorption period. No infectious virus was detected in supernatants from macrophages infected and cultured at 37 °C, although budding virus was observed by thin-section electron microscopy at this temperature.

Although infectious virus production was detected in cultures, this may have been due to replication in contaminating fibroblasts rather than in macrophages. An infectious centre assay was therefore employed to resolve this question. Assays were performed on cultures treated with trypsin since this was found to significantly reduce the number of contaminating fibroblasts (Table 1). The number of fluorescent foci per coverslip was found to be at least 100-fold greater than the number of contaminating fibroblasts (Table 1) and represented production of infectious virus by 2 to 4% of the cultured macrophages. No foci were observed when coverslips were treated with virus in the absence of macrophages.

#### DISCUSSION

The immunofluorescence, infectivity and electron microscopy studies described provide clear evidence for the replication of HCV 229E in cultured human macrophages *in vitro*. These findings are compatible with the relatively broad tissue tropisms exhibited by the coronavirus group (McIntosh, 1974). The morphogenesis and appearance of mature particles within the swollen cisternae of the endoplasmic reticulum closely correspond with the description given by Becker *et al.* (1967) for the growth of HCV 229E in WI-38 cells. In contrast to other coronaviruses, previous studies of HCV 229E have provided no evidence that the virus induces the formation of syncytia. The present report provides the first set of data showing virus-induced cell fusion by HCV 229E. Giant cells were observed by light and electron microscopy, and large multinucleate cells were seen by thin-section electron microscopy. The percentage of giant cells in the infected macrophage population was, however, relatively small compared with that found in studies of mouse hepatitis virus (MHV) 3-infected mouse macrophages; in this system giant cells can account for 50% of the infected cell population 24 h after infection (Virelizier & Allison, 1976).

The number of macrophages showing virus particles present in the endoplasmic reticulum was less than the number positive for virus antigen by immunofluorescence, but greater than

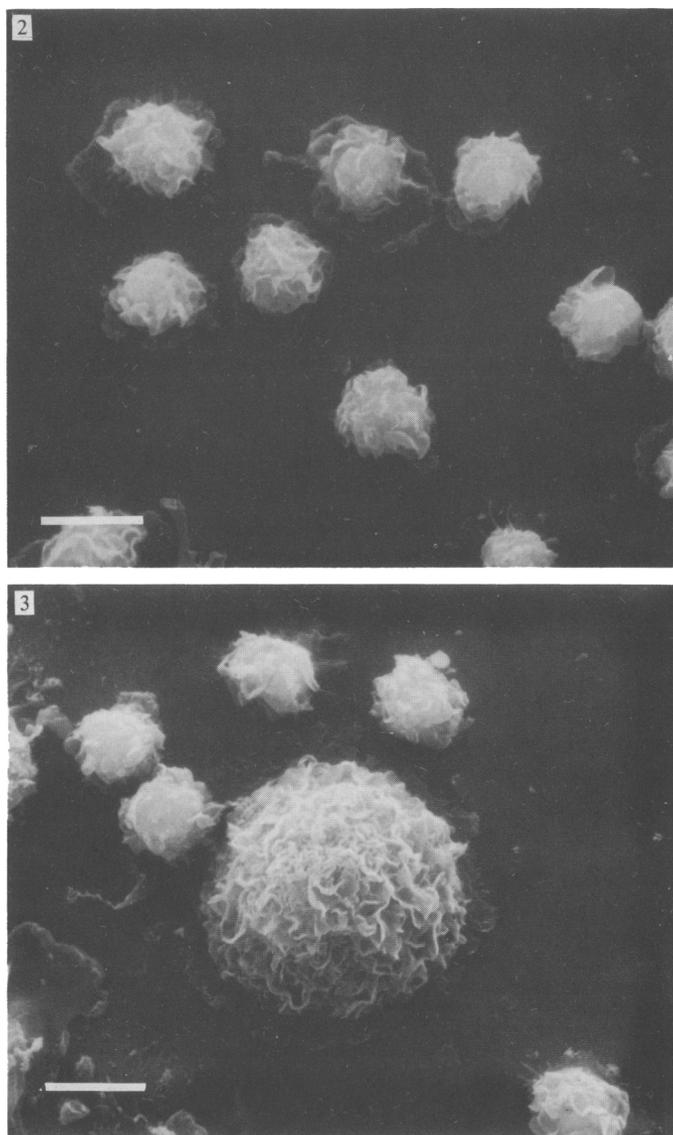


Fig. 2. Scanning electron micrograph of control uninfected human macrophages after 48 h in culture. Bar marker represents 10  $\mu\text{m}$ .

Fig. 3. Scanning electron micrograph of a giant cell found in a human macrophage culture 24 h after infection with HCV 229E. Bar marker represents 10  $\mu\text{m}$ .

the number of cells producing infectious virus as determined by infectious centre assay. The immunofluorescence results may indicate a block in some cells in the infectious cycle prior to virus assembly. Alternatively, a proportion of the macrophages may have escaped infection by the original virus inoculum and become infected later by virus released from infectious cells. At 24 h post-infection secondarily infected cells would be expected to contain newly synthesized virus antigen but not necessarily mature particles. The number of cells producing infectious virus compared with the number of virus-containing cells observed by electron microscopy may reflect the production of non-infectious virus. The observation of

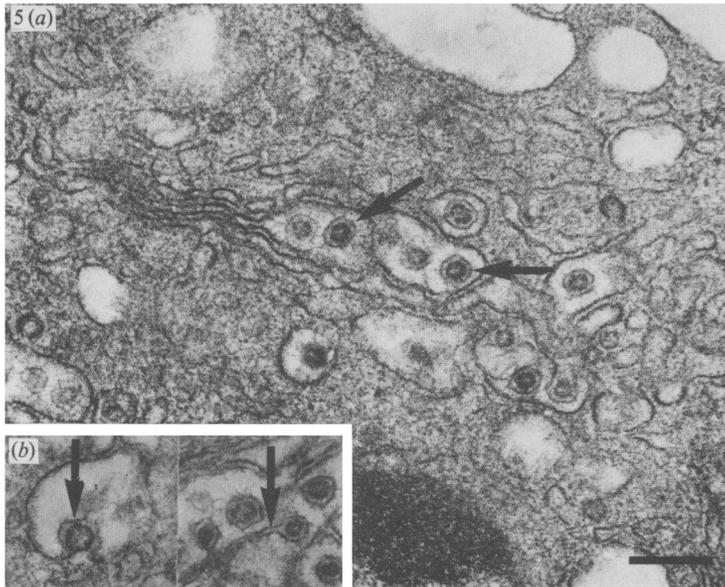
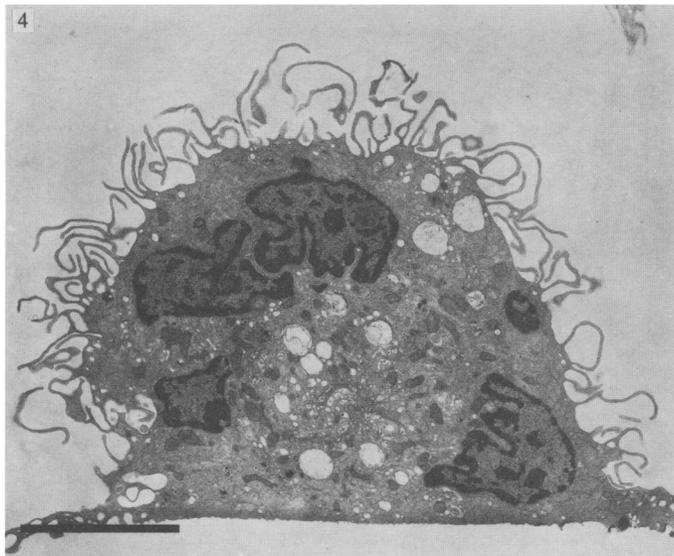


Fig. 4. Electron micrograph of a large multinucleate cell found in a human macrophage culture 24 h after infection with HCV 229E. Bar marker represents 5  $\mu$ m.

Fig. 5. Electron micrograph of a human macrophage 24 h after infection with HCV 229E. (a) Virus particles are present in the cisternae of the endoplasmic reticulum (arrows). (b) The inset shows virus particles in the process of budding (arrows) into the cisternae of the endoplasmic reticulum. Bar marker represents 200 nm.

Table 1. *Number of fluorescent foci in macrophage cultures infected with HCV 229E*

No. per coverslip	Expt. 1	Expt. 2
Macrophages*	33 500	58 200
Fibroblasts*	10	8
Fluorescent foci	1400	1200

\* Estimated by scanning electron microscopy.

virus budding into the endoplasmic reticulum in macrophages infected at 37 °C and the absence of infectious particles in these cultures strongly suggests that the production of non-infectious HCV 229E in macrophages can occur.

Macrophages are an important component in immune defence against viruses (Mims, 1964; Silverstein, 1970; Morgensen, 1979). A number of studies with different viruses has shown that the sensitivity of *in vitro* cultured macrophages to infection mirrors the sensitivity of the whole animal (Bang & Warwick, 1960; Roberts, 1964; Tosolini & Mims, 1971; Virelizier & Allison, 1976); our findings provide similar evidence for HCV 229E. However, care must always be taken in extrapolating from *in vitro* experiments to the *in vivo* situation. Employing MHV 3-susceptible and -resistant mouse strains, Virelizier & Allison (1976) showed a clear correlation in susceptibility to infection between *in vitro* cultured macrophages and the whole animal. On the other hand, Macnaughton & Patterson (1980) failed to find a similar clear-cut relationship. These results may reflect differences in culture conditions or virus preparations. In order to overcome these problems the macrophages from infected animals should be examined. Such studies are obviously difficult in humans but have been performed in mice infected with ectromelia virus (Roberts, 1963), lymphocytic choriomeningitis virus (Tosolini & Mims, 1971) and MHV 3 (S. Patterson & M. R. Macnaughton, unpublished results). In all these cases there is a good correlation between the *in vivo* susceptibility of macrophages and the susceptibility of the whole animal. Thus, although our results firmly establish the potential permissiveness of the *in vivo* human macrophage for the growth of HCV 229E, conclusive evidence is still lacking.

Not all patients' macrophages readily supported HCV 229E replication. The reason for this is not clear, but it may reflect differences in immune status, age and genetic composition of the individuals tested.

In addition to virus immunity, macrophages are also important in the development of the humoral antibody response (Feldman, 1972). If infection of human macrophages by HCV 229E does occur *in vivo* there may be impairment of the specific immune response. Infection of macrophages could also aid the spread of virus and, in this respect, it would be useful to know whether monocytes, the circulating precursors of macrophages, are susceptible to infection.

In conclusion, this report provides the first evidence of HCV 229E replication in human macrophages. In addition, data showing virus-mediated fusion are presented. In view of the importance of macrophages in defence against viruses, factors governing susceptibility of these cells to infection are currently in progress.

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#### REFERENCES

- BANG, F. B. & WARWICK, A. (1960). Mouse macrophages as host cells for the mouse hepatitis virus and the genetic basis of their susceptibility. *Proceedings of the National Academy of Sciences of the United States of America* **46**, 1065–1075.
- BECKER, W. B., MCINTOSH, K., DEES, J. H. & CHANOCK, R. M. (1967). Morphogenesis of avian infectious bronchitis virus and a related human virus (strain 229E). *Journal of Virology* **1**, 1019–1027.
- FAKHRI, O., AL-MONDHIRY, H., RIFAAT, U. N., KHALIL, M. A. & AL-RAWI, A. M. (1978). Output of peritoneal cells during peritoneal dialysis. *Journal of Clinical Pathology* **31**, 645–647.
- FELDMAN, M. (1972). Cell interactions in the immune response *in vitro*. V. Specific collaboration via complexes of antigen and thymus derived cell immunoglobulin. *Journal of Experimental Medicine* **136**, 737–760.
- GANGULY, R., MILUTINOVICH, J., LAZZELL, V. & WALDMAN, R. H. (1980). Studies of human peritoneal cells: a normal saline lavage technique for the isolation and characterization of cells from peritoneal dialysis patients. *Journal of the Reticuloendothelial Society* **27**, 303–310.

- KRAAJEVELD, C. A., MADGE, M. H. & MACNAUGHTON, M. R. (1980). Enzyme-linked immunosorbent assay for coronaviruses HCV 229E and MHV 3. *Journal of General Virology* **49**, 83-89.
- McINTOSH, K. (1974). Coronaviruses: a comparative review. *Current Topics in Microbiology and Immunology* **63**, 85-129.
- MACNAUGHTON, M. R. & MADGE, M. H. (1978). The genome of human coronavirus strain 229E. *Journal of General Virology* **39**, 497-504.
- MACNAUGHTON, M. R. & PATTERSON, S. (1980). Mouse hepatitis virus strain 3 infection of C57, A/Sn and A/J strain mice and their macrophages. *Archives of Virology* **66**, 71-75.
- MACNAUGHTON, M. R., THOMAS, B. J., DAVIES, H. A. & PATTERSON, S. (1980). Infectivity of human coronavirus strain 229E. *Journal of Clinical Microbiology* **12**, 462-468.
- MIMS, C. A. (1964). Aspects of the pathogenesis of virus diseases. *Bacteriological Reviews* **28**, 30-71.
- MONTO, A. S. (1974). Coronaviruses. *Yale Journal of Biology and Medicine* **47**, 234-251.
- MORGENSEN, S. C. (1979). Role of macrophages in natural resistance to virus infections. *Microbiological Reviews* **43**, 1-26.
- ROBERTS, J. A. (1963). Histopathogenesis of mousepox. III. Ectromelia virulence. *British Journal of Experimental Pathology* **44**, 465-472.
- ROBERTS, J. A. (1964). Growth of virulent and attenuated ectromelia virus in cultured macrophages from normal and ectromelia-immune mice. *Journal of Immunology* **92**, 837-842.
- SILVERSTEIN, S. (1970). Macrophages and viral immunity. *Seminars in Hematology* **7**, 185-214.
- TOSOLINI, F. A. & MIMS, C. A. (1971). Effect of murine strain and viral strain on the pathogenesis of lymphocytic choriomeningitis infection and a study of footpad responses. *Journal of Infectious Diseases* **123**, 134-144.
- VIRELIZIER, J. L. & ALLISON, A. C. (1976). Correlation of persistent mouse hepatitis virus (MHV3) infection with its effect on mouse macrophage cultures. *Archives of Virology* **50**, 279-285.

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