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13. We thank R. Bates, Department of Chemistry, University of Arizona, Tucson, for the interpretation of the ^{1}H NMR spectra; D. Penney, Department of Anatomy, University of Rochester, for the electron microscopic analysis; M. Kuroda, Department of Chemistry, University of Rochester, for running the NMR spectra; J. Leuenberger, F. Hoffmann–La Roche Co., Switzerland, for the samples of lutein and zeaxanthin; and D. L. Fox, University of California at San Diego, for suggesting yellow pigment might be lutein and the blue pigment might be biliverdin. This work was supported by grant PCM 77-07071 from the National Science Foundation, Bio. Med. research grant RR-07002, and a grant from the University of Arizona Foundation.

8 March 1982; revised 19 November 1982

Ammonium Chloride Prevents Lytic Growth of Reovirus and Helps to Establish Persistent Infection in Mouse L Cells

Abstract. *Ammonium chloride, a lysosomotropic agent that raises intralysosomal pH, reduces the yield of reovirus during infection of mouse L cells. Subsequent removal of ammonium chloride results in the rapid establishment of a persistent infection.*

Viruses as well as hormones and toxins may enter cells through receptor-mediated endocytosis (*1–3*). Since this pathway involves transport of viruses into lysosomes before viral entry into the cytoplasm, altering the lysosomal contents could affect the early interaction of viruses with cells (*4*). For example, treatment of cells with lysosomotropic weak bases such as NH_4Cl raises the intralysosomal *p*H (*5*), which in turn may inhibit fusion activity of enveloped viruses and block viral replication (*6, 7*). We report here that replication of the mammalian reoviruses—icosahedral, nonenveloped viruses containing segmented, double-stranded RNA—is inhibited by NH_4Cl. In addition, if NH_4Cl is removed after treating cells for 4 days in culture, persistent infection is readily established. Thus, the effect of NH_4Cl on viral replication is not limited to enveloped viruses, and persistent, noncytocidal infections may be readily established after such treatments.

To determine the effect of NH_4Cl on reovirus replication, virus was adsorbed to mouse L cells in monolayer at 37°C for 1 hour. The cells were then maintained in medium with or without 10 m*M* NH_4Cl. After 4 days the yield of reovirus type 1 or type 3 was more than 2 $\log_{10}$ lower in NH_4Cl-treated cells than in the controls (Fig. 1). In addition, reovirus-infected cells treated with NH_4Cl did not exhibit the characteristic cytopathic effects. Four days after infection, L cells not treated with NH_4Cl were rounded and dying; the treated cells, however, showed minimal cytopathic effects (Fig. 2). To determine whether this reduced destruction of L cells is correlated with increased cell viability, we exposed cells that had survived in the presence of NH_4Cl to trypan blue. Virtually all the cells excluded the dye, indicating that they were not only morphologically intact but also fully viable.

Since NH_4Cl markedly reduced the capacity of reovirus to damage L cells, we next sought to determine whether the infected cells could continue to replicate while supporting the growth of infectious virus. L cells were infected with reovirus type 1 and type 3 in the presence of NH_4Cl, and 96 hours later the medium was removed and replaced with fresh medium without NH_4Cl. By the following day (120 hours after infection) 50 to 75 percent of the cells had been lysed. However, 25 to 50 percent of the cells survived and a persistent infection was readily established. During the initiation of these cultures, titers of infectious virus increased from 10^7 to 10^8 plaque-forming units per milliliter (Fig. 1). By day 10 the cells were doubling every 48 hours (compared to 24 hours for parental L cells). The medium for these cells contained 10^6 to 10^8 plaque-forming units of reovirus per milliliter for at least 98 days after the initiation of the persistent infection. The persistently infected cell lines are stable and have been passaged more than 30 times in 4 months. Ten different persistently infected lines have been established by means of this approach. Thus, removal of NH_4Cl after 4 days of infection allowed virus titers to increase initially and routinely resulted in persistently infected cell cultures.

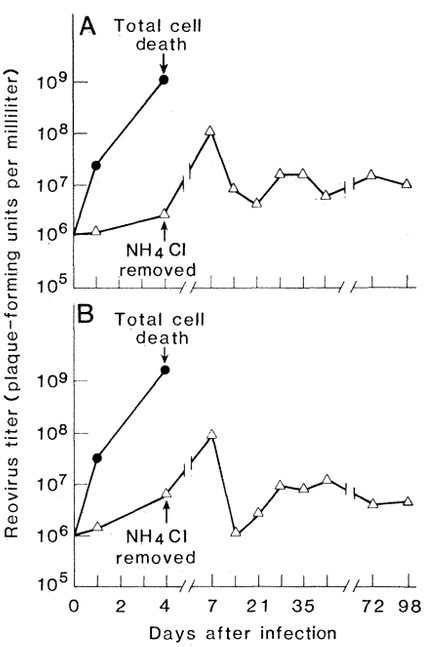

Fig. 1. Effect of NH_4Cl on the yield of reovirus type 1 (A) or type 3 (B) in mouse L cells. Virus was adsorbed to mouse L cells at a multiplicity of infection of 1 at 37°C for 1 hour in the absence of NH_4Cl. The cells were then overlaid with Joklik's modified Eagle's medium supplemented with 5 percent fetal calf serum with (△) or without (●) 10 m*M* NH_4Cl. By day 4 after infection there was complete cell lysis of cells infected in the absence of NH_4Cl. On day 4 the supernatant was removed from the treated cells and replaced with fresh medium not containing NH_4Cl. These cells were then maintained in NH_4Cl-free medium. Virus was grown, maintained, and titered in mouse L cell monolayers (*18*).

To further characterize the nature of the persistent infection, we analyzed cells by infectious center assay, immunofluorescence, and electron microscopy. Infectious center assay showed that 20 to 40 percent of the cells were producing infectious virus. Reovirus antigens were detected in a subset of the persistently infected cells by immunofluorescence (Fig. 3). Many cells expressed little or no viral antigen, while others were strongly fluorescent. Overall, about 25 percent of the cells were positive for viral antigen. An electron micrograph of representative antigen-positive cells (Fig. 3C) shows crystalline arrays of reovirus within cytoplasmic inclusions. Thus, the persistently infected cell lines that were started in cultures treated with NH_4Cl were similar to other reovirus carrier cultures in that they contained readily detectable viral inclusions and released large amounts of infectious virus (*8, 9*). The high percentage of cells containing no detectable viral antigens did, however, differ from our previous results that indicated a proportion of antigen-positive cells of 80 to 100 percent in carrier cultures established after infection with defective viruses (*9*).

Some investigators have argued that NH_4Cl, by raising intralysosomal *p*H, inhibits a low *p*H-dependent fusion activity of enveloped viruses in the lysosome, blocking viral penetration of the

cytoplasm (*7*). Others have suggested that amines such as NH_4Cl inhibit viral entry by blocking uptake at the cell surface (*10, 11*). To preclude the latter possibility in the present study, we adsorbed reovirus to the cells at 37°C for 1 hour before adding NH_4Cl. Since reovirus uptake is nearly complete 1 hour after inoculation (*12*), NH_4Cl probably does not act by preventing viral uptake. Furthermore, since reovirus is an icosahedral, nonenveloped virus with no known fusion activity, an alteration in lysosomal proteases may be responsible for blocking reovirus uncoating and entry or a penetration activity in the reovirus replicative cycle may be affected by NH_4Cl (*13*).

In addition to inhibiting the replication of reovirus under conditions of acute infection, removal of NH_4Cl from NH_4Cl-treated, infected cells resulted in the establishment of persistently infected cell cultures. Persistent infections have usually been initiated with defective (*DI*) or highly mutated (*ts*) viruses (*14, 15*). In facilitating the establishment of persistent infection, NH_4Cl may act primarily on the cell or on the virus. We do not know whether abnormal viruses are generated by treatment of cells with NH_4Cl. It is possible, for example, that defective, interfering particles or temperature-sensitive viral mutants may be rapidly generated in cells so treated. Alternatively, NH_4Cl may exert its primary effect on the cell, leading to phenotypic protection of certain subpopulations of cells while facilitating the emergence of a wide range of virus-resistant cells capable of becoming persistently infected. The relatively low percentage of cells containing viral antigen and the observation that not all cells contain infectious virus argue that virus-resistant cells emerge during persistent infection (*16*).

The establishment of persistent infections in cells in vitro following the use of NH_4Cl may have important implications for the use of lysosomotropic drugs in vivo. For example, amantadine hydrochloride, a lysosomotropic weak base, is currently prescribed for the prevention and treatment of influenza A virus infections (*17*). The use of lysosomotropic agents as antiviral drugs may theoretically enhance persistent viral infections in clinical settings.

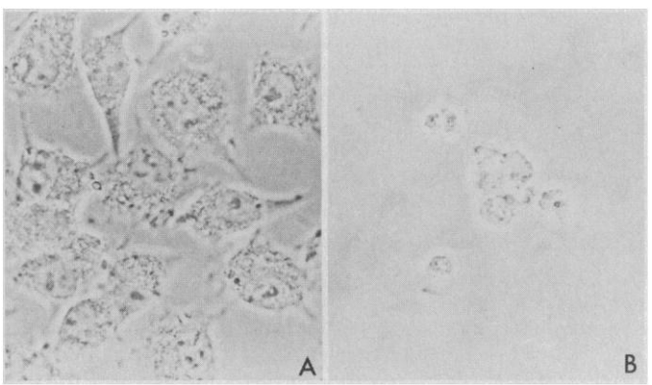

Fig. 2. Light microscopic appearance of mouse L cells infected with type 3 reovirus in the presence (A) or absence (B) of 10 m*M* NH_4Cl. L cells were infected with reovirus at a multiplicity of infection of 1 and maintained in minimum essential medium with or without 10 m*M* NH_4Cl. Four days after infection the cells were fixed in acetone and examined by light microscopy.

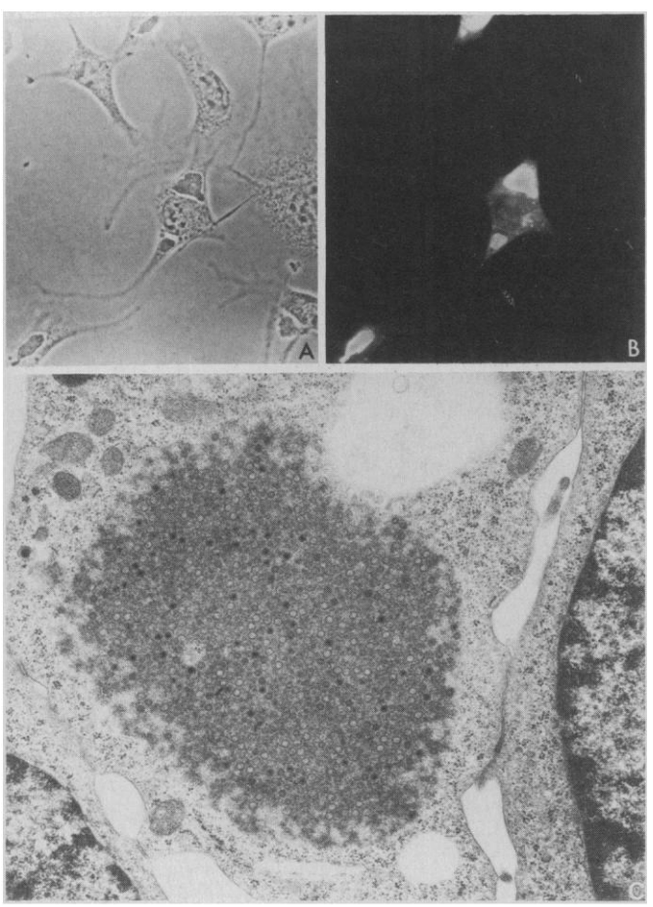

Fig. 3. Presence of reovirus in persistently infected cells. Mouse L cells were treated with 10 m*M* NH_4Cl during the first 4 days after infection with reovirus. The NH_4Cl was removed on day 4 and the cells were subsequently maintained in medium without it. Phase-contrast and reovirus immunofluorescence of type 3 cells on day 37 after infection are shown in (A) and (B), respectively. Indirect immunofluorescence to detect the presence of reovirus proteins was 100 percent positive in mouse L cells acutely infected with reovirus and completely negative in uninfected mouse L cell controls. (C) Electron microscopic view of a reovirus inclusion body in a persistently infected type 3 cell.

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19. We gratefully acknowledge the technical help of E. Freimont and the skillful typing of J. Segal. We also thank R. Ahmed, E. Brown, R. Kauffman, A. Sharpe, and D. Knipe for helpful discussions, and J. Robinson for superb electron microscopy. W.M.C. is a Carl Walter Fellow at Harvard Medical School. Supported by NIH grant AI 13178.

28 July 1982; revised 10 September 1982