

## Calcium Requirement for Syncytium Formation in HEp-2 Cells by Respiratory Syncytial Virus

MAHMOUD SHAMSI SHAHRABADI<sup>1,2\*</sup> AND PATRICK W. K. LEE<sup>1</sup>

*Department of Microbiology and Infectious Diseases, University of Calgary Health Sciences Centre,<sup>1</sup> and Foothills Provincial Hospital,<sup>2</sup> Calgary, Alberta, Canada T2N 4N1*

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**Respiratory syncytial virus (RSV) grown in HEp-2 cells in the absence of calcium did not induce cell fusion and syncytium formation. Although the infected cells contained viral antigens, the cytopathic effect (giant cell formation) typical for RSV was not observed in calcium-free cultures. Infectious virus yield was also slightly reduced (less than a one log<sub>10</sub> reduction) in the absence of calcium. An analysis of viral proteins synthesized in both the presence and the absence of calcium revealed that the amount of fusion protein (F<sub>1</sub>) in calcium-free infected cultures was approximately one-third that in calcium-containing infected cultures. These results underscore the necessity of using calcium-containing growth medium for cell culture isolation and diagnosis of RSV.**

The most important viral infection of the lower respiratory tract in infants and children is caused by respiratory syncytial virus (RSV) (4). The virus is composed of a nucleocapsid containing a single-stranded RNA genome. The nucleocapsid is surrounded by a bilayer membrane with short, closely spaced projections. It is believed that the viral genome codes for at least eight viral structural proteins (5, 11), two of which are glycosylated (2, 3). The major viral surface proteins are the G protein, which recognizes and attaches to the host cell receptor, and the F protein (molecular weight, 68,000 to 70,000), which mediates the fusion of the viral envelope with the host cell membrane (15). In infected cells, the F glycoprotein is cleaved by proteolytic enzymes to yield two smaller proteins, F<sub>1</sub> (molecular weight, 48,000) and F<sub>2</sub> (molecular weight, 20,000), which are assembled into the mature virion (7, 9). The most distinct morphological change induced by RSV in cultured cells is the fusion of infected cells with neighboring infected or uninfected cells, resulting in syncytium formation (1). We report here that cell fusion and syncytium formation by RSV are calcium dependent.

HEp-2 cells were grown as monolayers in minimal essential medium (MEM) containing 4% fetal bovine serum. RSV (Long strain) was obtained from the American Type Culture Collection, Rockville, Md. HEp-2 cells were infected with RSV at a multiplicity of 5 50% tissue culture infective doses per cell. After 1 h of incubation, the inoculum was removed, and the cells were washed three times with phosphate-buffered saline and incubated in calcium-free MEM containing 1% dialyzed fetal bovine serum. As controls, cells were infected similarly and incubated in regular MEM containing 1% dialyzed fetal bovine serum. At 48 h postinfection, cells were fixed in acetone at 4°C for 10 min and stained by the indirect immunofluorescence technique with RSV antisera obtained from the Wellcome Foundation, Beckenham, England.

Syncytium formation was detected in cells grown in the presence of calcium, and viral antigens were present in the cytoplasm of these multinucleated giant cells (Fig. 1A). In contrast, infected cells grown in the absence of calcium did not fuse, although they contained cytoplasmic RSV antigens (Fig. 1B). These cells remained separated, became rounded

at the late stage of infection, and were therefore morphologically distinct from infected cells maintained in the presence of calcium.

To determine the effect of calcium deprivation on virus yield, we harvested infected cells grown in the presence or absence of calcium daily and determined the titer of infectious virus by the 50% tissue culture infective dose method by using the Reed-Muench calculation (12). The yield of infectious virus was not drastically affected by calcium deprivation (Fig. 2). There was less than a one log<sub>10</sub> reduction in the titer of infectious virus grown in calcium-free medium as compared with the control.

To analyze the viral structural proteins synthesized in the absence of calcium, we labeled infected cells grown in the presence or absence of calcium with [<sup>35</sup>S]methionine (10 μCi/ml) at 6 h postinfection and harvested them 4 days later. The cells were combined with the supernatant culture medium and centrifuged at 25,000 rpm for 2 h at 4°C (SW28 rotor, L-70 ultracentrifuge; Beckman Instruments, Inc., Fullerton, Calif.). The pellet was suspended in 1% Triton X-100 in phosphate-buffered saline. After 15 min of incubation on ice with frequent vortexing, the nuclei were sedimented in a Microfuge (Beckman), and the supernatant was used for radioimmunoprecipitation. A 50-μl quantity of a 1/10 dilution of commercially available RSV antisera (Wellcome) was mixed with 50 μl of a Triton X-100 extract of infected cells and incubated at room temperature for 30 min. The antigen-antibody complex was precipitated with fixed *Staphylococcus aureus* and electrophoresed on a 10% polyacrylamide gel by the method of Laemmli (6). Resolved proteins were visualized by fluorography. The position of each protein band was determined by comparing the gel pattern with that previously reported (5). The molecular weight of each protein was estimated with rotavirus proteins as markers. At least nine major structural proteins were detected in infected cells grown in the presence or absence of calcium (Fig. 3). However, the quantity of F<sub>1</sub> protein present in the calcium-deprived culture was much lower than that in the control (calcium-containing) culture. None of the other proteins appeared to be affected by calcium deprivation. (The levels of F and F<sub>2</sub> in the two cultures were too low to allow for the detection of any differences.) To compare the amounts of F<sub>1</sub> protein present in the two cultures, we

\* Corresponding author.

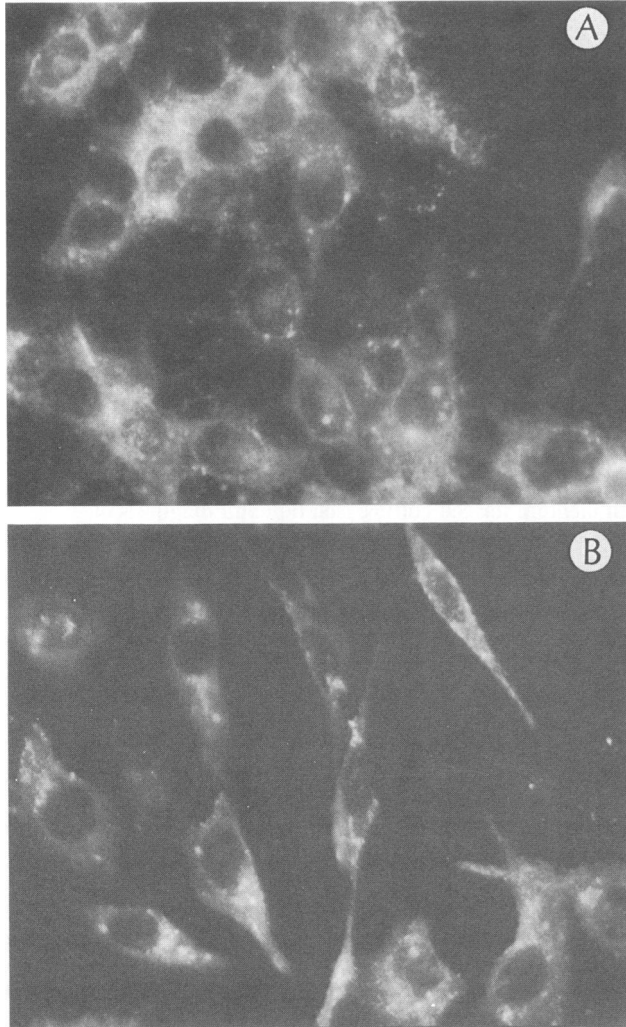


FIG. 1. (A) HEP-2 cells infected with RSV and maintained in regular MEM for 48 h. Multinucleated fused cells containing viral antigens inside the cytoplasm are visible. (B) HEP-2 cells infected with RSV and maintained in calcium-free MEM for 48 h. The cells contained viral antigens. No syncytium formation could be detected. Cells in both panels were stained by the indirect immunofluorescence method.

subjected the fluorograph to scanning laser densitometry (model 202 densitometer; LKB Instruments, Inc., Rockville, Md.). The amount of  $F_1$  protein in the calcium-deprived culture was approximately one-third that in the control culture (data not shown). This difference was much less pronounced when protein profiles of purified virions from the two cultures were compared (data not shown). Thus, virus assembly appears to proceed normally in the absence of calcium. However, calcium deprivation probably affects the synthesis or stability of the  $F_1$  protein, resulting in a slight reduction in virus yield. No major differences in viability or protein pattern could be detected between uninfected HEP-2 cells maintained in the presence or absence of calcium (data not shown). The omission of other divalent cations (e.g., magnesium) from the medium did not have any apparent effect on the cytopathic effect of RSV.

It has been reported that glutamine plays a critical role in RSV-induced syncytium formation (8). In the absence of

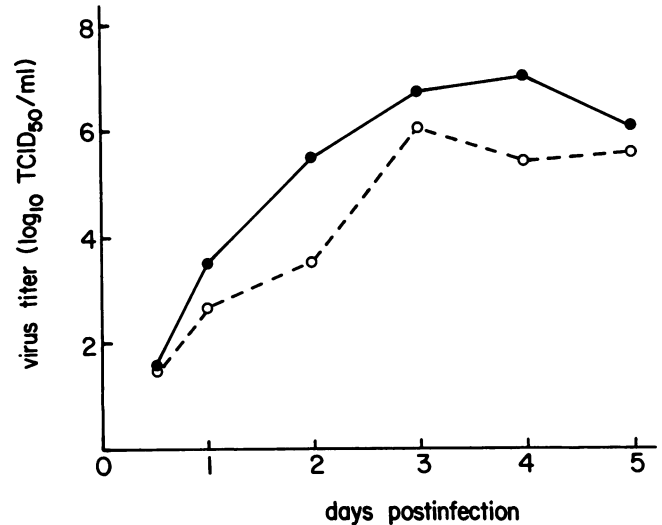


FIG. 2. Infectious virus titer. Cells infected with RSV in both the presence (●) and the absence (○) of calcium were harvested daily, and the titer of infectious virus was determined. TCID<sub>50</sub>, 50% Tissue culture infective dose.

glutamine, cell fusion and multinucleated giant cell formation were blocked. In addition, the yield of infectious virus in glutamine-free cultures was considerably reduced (8). In our study, infectious virus production did not seem to be affected significantly. It thus appears that cell fusion and syncytium formation are not required for virus multiplication. In other virus-cell systems, such as rotavirus and cytomegalovirus, calcium plays an important role in the process of viral replication and virus assembly (10, 13, 14). The fusion observed in rotavirus-infected cells is inhibited in the absence of calcium, and the maturation of infectious

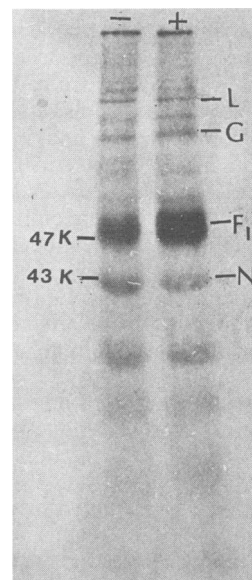


FIG. 3. RSV proteins synthesized in the presence (+) or absence (-) of  $\text{CaCl}_2$ . [ $^{35}\text{S}$ ]methionine-labeled infected cells were harvested 4 days postinfection and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Molecular weights in thousands (K) are shown at left; proteins are shown at right.

virus is blocked by calcium deprivation (14). Although the significance of syncytium formation in the pathogenesis of RSV infections is not clear, the morphological changes induced in infected cells are significant criteria for the isolation and diagnosis of RSV in cultures. The exact role of calcium in the synthesis of viral proteins and virus assembly remains to be determined.

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#### LITERATURE CITED

1. Chanock, R. M., B. Roizman, and R. Myers. 1957. Recovery from infants with respiratory illness of a virus related to chimpanzee coryza agent. I. Isolation, properties and characterization. *Am. J. Hyg.* **66**:281-290.
2. Dubovi, E. J. 1982. Analysis of proteins synthesized in respiratory syncytial virus-infected cells. *J. Virol.* **42**:373-378.
3. Fernie, B. F., and J. L. Gerin. 1982. Immunochemical identification of viral and nonviral proteins of the respiratory syncytial virus virion. *Infect. Immun.* **37**:243-249.
4. Glezen, W. P., and F. W. Denney. 1973. Epidemiology of acute lower respiratory disease in children. *N. Engl. J. Med.* **288**:498-505.
5. Huang, Y. T., P. L. Collins, and G. W. Wertz. 1985. Characterization of the 10 proteins of human respiratory syncytial virus: identification of a fourth envelope-associated protein. *Virus Res.* **2**:157-173.
6. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London)* **227**:680-685.
7. Lambert, D. M., and M. W. Pons. 1983. Respiratory syncytial virus glycoproteins. *Virology* **130**:204-214.
8. Marquez, A., and G. D. Hsiung. 1967. Influence of glutamine on multiplication and cytopathic effect of respiratory syncytial virus. *Proc. Soc. Exp. Biol. Med.* **124**:95-99.
9. McIntosh, K., and R. M. Chanock. 1985. Respiratory syncytial virus, p. 1285-1304 *In* B. N. Fields, D. M. Knipe, R. M. Chanock, J. L. Melnick, B. R. Roizman, and R. E. Shope (ed.), *Virology*. Raven Press, Publishers, New York.
10. Notka, D., O. S. Eaton Steinsland, and T. Albrecht. 1987. Ca<sup>2+</sup> responses in cytomegalovirus-infected fibroblasts of human origin. *Virology* **157**:259-267.
11. Pringle, C. R., P. V. Shirodaria, H. B. Gimenz, and S. Levine. 1981. Antigen and polypeptide synthesis by temperature sensitive mutants of respiratory syncytial virus. *J. Gen. Virol.* **54**:173-183.
12. Reed, L. J., and H. Muench. 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* **27**:493.
13. Shahrabadi, M. S., L. A. Babiuk, and P. W. K. Lee. 1987. Further analysis of the role of calcium in rotavirus morphogenesis. *Virology* **158**:103-111.
14. Shahrabadi, M. S., and P. W. K. Lee. 1986. Bovine rotavirus maturation is a calcium-dependent process. *Virology* **152**:298-307.
15. Walsh, E. E., and J. Hruska. 1983. Monoclonal antibodies to respiratory syncytial virus proteins: identification of the fusion protein. *J. Virol.* **47**:171-177.