Adaptation of Respiratory Syncytial (RS) Virus to Brain of Suckling Mice. (30691)

JOSEPH J. CAVALLARO* AND HUNEIN F. MAASSAB†
(Introduced by Thomas Francis, Jr.)
Department of Epidemiology, School of Public Health, University of Michigan, Ann Arbor

Since its first isolation in 1956-1957(1,2) the importance of respiratory syncytial (RS) virus in acute respiratory disease has increased and today it is considered to be one of the most important causes of respiratory illness in infants(3,4). Efforts directed towards the development of a vaccine against RS virus are now in progress(4). Because of the adaptation of RS virus to a susceptible laboratory animal would prove valuable as an assay system for the *in vivo* effectiveness of biological and/or therapeutic agents affecting the virus, an attempt was undertaken to adapt the virus to mice. The present study indicates that RS virus may be propagated in suckling mice and will report on two separate serial intracerebral passage series in these animals.

Materials and methods. Virus. The Long strain of respiratory syncytial virus, isolated in 1957 by Chanock *et al*(2) was used in this study for the initial inoculation of the mice. The virus had been passed in our laboratory in tissue cultures of HL cells.

Tissue culture. HL cells, a continuous line of human epithelial cells derived in our laboratory, were grown in 2X Eagle's medium containing 5% inactivated calf serum together with a standard antibiotic solution of penicillin and streptomycin. Prior to inoculation these media were replaced with maintenance media consisting of 2X Eagle's with 3% inactivated horse serum.

Inoculation of mice. Laboratory-bred Webster albino mice were used within 24 hours after birth for the serial passage of the virus *via* the intracerebral route. At the initiation of the passage series, 8 infant mice were inoculated intracerebrally with 0.03 ml of undiluted RS virus infected tissue culture fluid. Thereafter, either a 20% or 10% infected mouse brain suspension or dilutions thereof was used as inoculum for the succeeding passages. Control mice were inoculated with like amounts of a sterile 10% suspension of normal mouse brain. The control mice were initially inoculated with undiluted non-infected tissue culture fluid.

Virus recovery. Mice were sacrificed 6 days after inoculation and the brain tissue removed aseptically and a 20% suspension prepared in sterile diluent by grinding in a mortor with alundum. The suspension was centrifuged in the cold at 2500 rpm for 10 minutes and the supernatant fluid collected. The supernatant was quickly frozen in glass sealed ampules in an alcohol-dry ice bath and stored in dry-ice chest for infectivity titrations. Normal control mice were similarly sacrificed and 20% brain suspensions prepared.

Virus assay. Serial 10-fold dilutions of the brain suspensions were back titrated in HL cells; 0.1 ml of each dilution was inoculated into each of 4 HL tubes. The culture tubes were fed every third day and held for a maximum of 14 days, at which time the $TCID_{50}$ per ml was calculated by the method of Reed and Muench(5).

For *in vivo* titrations, suckling mice were inoculated intracerebrally with 0.03 ml of 10-fold dilutions of the infected brain suspensions. At 14 days post inoculation, titers based on death as the endpoint (LD_{50}) were calculated.

Serum neutralization tests. The immune serum employed in the neutralization tests was produced in adult ferrets by immunization with the Long strain of RS virus which had been passed in BS-C-1 cell cultures(6). Ferrets were inoculated by the intranasal

* Supported in part by a Graduate Training Grant for microbiologists from Nat. Inst. of Allergy and Infect. Dis., NIH, 2 T1 AI-60 and 5 T1 AI-60.

† Conducted in part under auspices of Commission on Influenza, Armed Forces Epidemiological Board, and supported in part by Office of Surgeon General, U. S. Army, Washington, D.C.

route with 0.5 ml of the undiluted tissue culture virus. They were bled 3 weeks after inoculation and also received, by the intraperitoneal route, a booster inoculation of 1.0 ml of undiluted virus. Immune sera were obtained 3 weeks after the final inoculation. The ferret serum demonstrated a neutralization titer of 1:256.

The normal and immune sera were inactivated at 56°C for 30 minutes before mixing with the virus. In addition, specific immune horse serum‡ with a neutralization titer of 1:640, was also used in the neutralization tests. The virus-serum mixtures were held at room temperature for one hour. A standard inoculum of 0.2 ml was added to HL cell culture tubes containing 1.0 ml of media. The tubes were observed daily for 14 days(7).

For neutralization tests in suckling mice, the immune and normal sera, inactivated as above, were prepared at a final dilution of 1:16 with 10% infected mouse brain suspensions; 0.03 ml of the virus-serum mixture was inoculated intracerebrally into the mice. Control inoculum consisted of a 1:2 dilution of 10% infected mouse brain suspension.

Direct fluorescent antibody staining. Crude globulin fractions of immune ferret serum were prepared by half saturation with ammonium sulfate. Globulin solutions were conjugated with fluorescein isothiocyanate(8) and the conjugates fractionated by the cellulose anion exchange method(9).

Staining was carried out by the direct method(10) on both infected and non-infected HL cell cultures grown on coverslips in Leighton tubes. Preliminary staining was done in cells infected with the Long strain of RS virus to evaluate the specificity of the conjugated serum. Fluorescence was not seen, when coverslips, at various stages of infection, were treated with conjugated normal ferret serum. Likewise, fluorescence was not observed when non-infected coverslips were treated with labelled immune serum.

Results and conclusions. In attempting to adapt RS virus to suckling mice, the virus was tested initially for its ability to multiply in the lungs of these animals. Towards this end the virus was administered by the intranasal, intrathoracic and intraperitoneal routes. Attempts to pass RS virus by these routes were not successful as the virus could not be recovered from lung tissue(s) after 3 serial passages. However, it was noted that the virus could be recovered after serial intracerebral passage and efforts were directed towards this route of inoculation.

First intracerebral mouse passage series. The titer of the tissue culture virus used to initiate the first passage series was $10^{-6.4}$ $TCID_{50}$ per ml. Early in this series, mice inoculated i.c. with infected brain suspension material failed to show signs of infection. However, the virus persisted in the brain as evidenced by back titration in HL cell cultures.

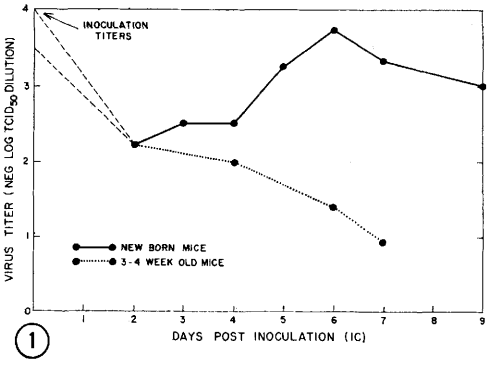
Fig. 1 presents results of titrations of the virus content of the brains of mice sacrificed at set intervals after i.c. inoculation with infected brain suspension from the 23rd passage. Examination of the data reveals that after inoculation, the amount of virus demonstrable in the brain diminished during the first 2 days to a level approximately one $\log_{10}$ below that of the inoculum. Between the third and fourth days the titer began to rise and reached its maximum level at the sixth day ($10^{-3.8}$ $TCID_{50}$ per ml) after which it began to decrease slowly.

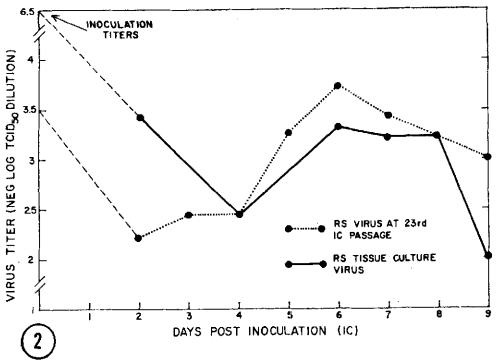
At the 34th passage in suckling mice, unsuccessful efforts were made to pass the virus intracerebrally in adult mice; as seen in Fig. 1, the virus failed to increase or persist in these mice.

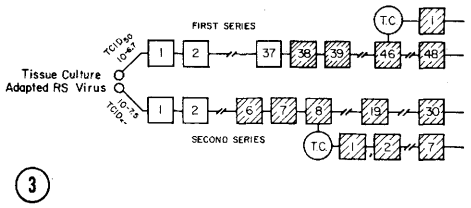
Comparison of the *in vivo* pattern of growth of the 23rd mouse passage virus with that of the tissue culture virus from which it was derived showed an overall similarity in growth curves, but with certain differences (Fig. 2). (1) The mouse passage virus initially exhibited a one $\log_{10}$ decrease in infectivity, whereas the tissue culture virus showed a decrease of 3 $\log_{10}$s; (2) the mouse passage virus persisted at a higher titer—day 9—than the tissue culture virus.

The first evidence of any pathologic effect was seen at passage 37 when 4 of the inoculated mice appeared ill and ataxic at 6 days

‡ Purchased from Flow Laboratories, Inc., Rockville, Md.







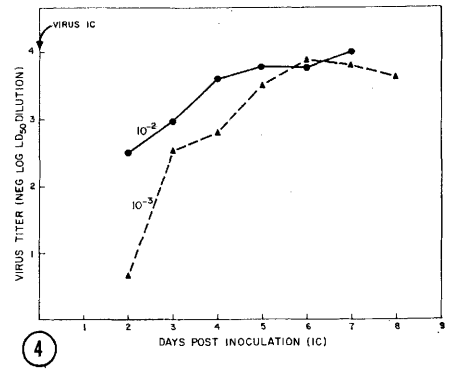


FIG. 1. Intracerebral growth of adapted RS virus in suckling mice and 3-4-week-old mice.
FIG. 2. Comparison of intracerebral growth in suckling mice of adapted and non-adapted RS virus.
FIG. 3. Schematic summary of adaptation procedure followed in the 2 serial passage series. Open blocks = no overt pathology or deaths; RS virus back-titrated in HL cell cultures. Shaded blocks = passage levels at which deaths occurred.
FIG. 4. Growth curves of virus (as measured by deaths) in brains of suckling mice inoculated with 2 concentrations of adapted RS virus.*

* From 29th passage of second series.

post-inoculation (Fig. 3). Passage 38 produced 2 deaths (shaded blocks) and in the succeeding passages, the deaths of mice were scattered between 6 and 14 days post-inoculation. The adapted virus reached a titer of between $10^{-2.5}$ and $10^{-3.0}$ LD_{50}, and the per cent mortality ranged from 35% to 85%. At passage 46 the adapted virus, recovered in HL culture tubes, was again passed intracerebrally for 2 passages and maintained its pathologic effect.

Second intracerebral mouse passage. To confirm the findings of the first passage series, a second series was initiated with a later tissue culture passage virus of higher titer ($10^{-7.6}$ $TCID_{50}$ per ml) than the first series.

The first evidence of a pathologic effect was seen at passage 6 (Fig. 3). At the 8th passage, 3 of 8 mice died 6 days after inoculation, with the remainder dying by the 12th day. In the succeeding passages 100% mortality was observed by the 14th day post-inoculation. As in the first series, the adapted virus could be passed intracerebrally after recovery in HL cell cultures, and maintained its pathologic effect.

Data obtained comparing the growth of adapted RS virus in the brains of mice by death after i.c. inoculation of 2 concentrations of the virus are shown in Fig. 4. Following the injection of 10^{-2} and 10^{-3} dilutions of the virus, a lag period of similar duration was noted for each concentration; however, once the titers began to rise on the 3rd day, they increased at approximately the same rate to reach maximal heights at the 5th to 6th days post-inoculation.

Virus induced effects. The infected mice

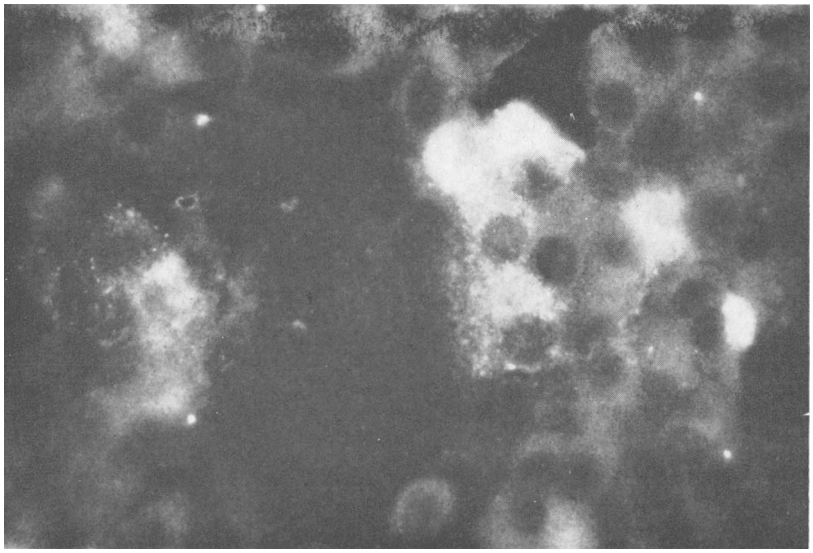


FIG. 5. Cytoplasmic antigen and early syncytial formation demonstrated by immunofluorescent staining of respiratory syncytial virus (RS) in HL cells inoculated with 10% suspension of infected mouse brain.

were found to be smaller in size than control mice of the same age and exhibited ataxic-like movements and incoordination; paralysis was not observed. At autopsy, the brain tissue in the RS virus inoculated mice appeared softer and more friable than normal. A histopathologic study of brain and other tissues from inoculated mice is now in progress.

Identification of mouse passage virus. Identification of the mouse passage virus(es) was made by means of (1) serum neutralization tests in HL cell cultures; (2) serum neutralization tests in suckling mice; (3) direct fluorescent antibody staining; and (4) observation of the characteristic cytopathic effect produced in HL cell cultures.

Serum neutralization tests in tissue culture were performed with specific immune serum prepared in ferrets; for control purposes normal ferret serum was employed. The immune serum completely inhibited the cytopathic effect of the virus, whereas the normal serum showed no significant inhibitory effect.

Serum neutralization tests in suckling mice were performed at various intervals following the inception of the pathologic effects. Table I presents the data obtained in typical neutralization experiments. Ferret immune serum at a dilution of 1:16 protected 88% and 100% of the mice; immune horse serum protected 100% of the mice at a dilution of 1:16. Normal serum showed no significant effects.

Direct fluorescent antibody staining studies were carried out on both infected and non-infected HL cells grown on coverslips in Leighton tubes. The tubes were inoculated with 0.1 ml of 10% infected brain suspensions and at various intervals coverslips were stained for RS virus antigen. In all instances antigen was seen localized to the cytoplasm and appeared as finely dispersed particles or in later stages as large syncytial formations (Fig. 5). Intranuclear fluorescence was not observed; this is in agreement with the results of tissue culture studies of RS virus reported by Kisch *et al*(11).

Summary. Respiratory syncytial virus (Long strain) was adapted to growth in suckling mice through 2 separate serial intra-

TABLE I. Identification of Mouse Passage RS Virus by *in vivo* Serum Neutralization Tests.

Virus passage	Per cent mortality		
	Normal serum	Immune serum	Control virus
42	72*	12*	53
R-18	100*	0*	100
R-31	100†	0†	100

R = Second passage series.
* Ferret serum.
† Horse serum.

cerebral passage series. The virus(es) produced a lethal effect in the animals 6 to 14 days following inoculation. Identification of the mouse passage virus was established by serum neutralization tests in tissue culture and in suckling mice. Further identification was established by direct fluorescent antibody staining of the mouse passaged virus after one passage in tissue culture. It is suggested that the growth of RS virus in brain of suckling mouse with resultant illness and death may have practical value in the evaluation of prospective vaccines.

1. Morris, J. A., Blount, R. E., Jr., Savage, R. E., Proc. Soc. Exp. Biol. and Med., 1956, v92, 544.

2. Chanock, R. M., Roizman, B., Myers, R., Am. J. Hyg., 1957, v66, 281.

3. Hilleman, M. R., Hamparian, V. V., Ketler, A., Reilly, C. M., McClelland, L., Cornfield, D., Stokes, J., JAMA, 1962, v180, 445.

4. Coates, H. V., Chanock, R. M., Postgrad. Med., 1964, v35, 460.

5. Reed, L. J., Muench, H., Am. J. Hyg., 1938, v27, 493.

6. Hopps, H. E., Bernheim, B. C., Nisalk, A., Tjio, J. H., Smadel, J. E., J. Immunol., 1963, v91, 416.

7. Hamre, D., in Diagnostic Procedures for Viral and Rickettsial Diseases, E. H. Lennette, N. J. Schmidt, eds., 1964, 3rd ed., p517.

8. Marshall, J. D., Eveland, W. C., Smith, C. W., Proc. Soc. Exp. Biol. and Med., 1958, v98, 898.

9. Riggs, J. L., Loh., P. C., Eveland, W. C., ibid., 1960, v105, 655.

10. Coons, A. H., Kaplan, M. H., J. Exp. Med., 1950, v91, 1.

11. Kisch, A. L., Johnson, K. M., Chanock, R. M., Virology, 1962, v16, 177.

Received July 26, 1965. P.S.E.B.M., 1966, v121.

Concentration of Complement Fixing Viral Antigens.* (30692)

KLAUS SCHELL, ROBERT J. HUEBNER, HORACE C. TURNER

Microbiological Associates, Inc., and Laboratory of Infectious Diseases, NIAID, National Institutes of Health, Bethesda, Md.

Pereira and Valentine(1), Wilcox and Ginsberg(2), and many others showed that cell-associated infective virus could be concentrated by slow speed cell sedimentation prior to release from the virus-bearing cells. Others(3,4) used this procedure to concentrate neo-antigens(5). In this note we describe its application for the routine preparation of high titered complement fixing (CF) antigens for a great variety of viruses.

The following preparations were made using standard virus inocula and standard virological and tissue culture procedures. No steps were taken to define carefully maximum virus propagation. The results, therefore, should not be regarded as optimal but rather as indicative of the usefulness of this technique.

Materials and methods. Cell cultures were obtained from Microbiological Associates, Inc. (Table I) and maintained on Eagle's Minimum Essential Medium in Earle's Balanced Salt Solution containing varying concentrations of Agamma Calf Serum (Hyland†), 4 mM of Glutamine, 100 Units of Penicillin and 100 γ of Streptomycin/ml.

Viruses were obtained from various sources; they were passed once or twice in our laboratory under recommended conditions in order to obtain standard seed stocks(6,7).

CF tests were done in the microtiter system (Cooke Engineering‡) as described by Sever (8): Antigens and control antigens were diluted serially in 0.025 ml volumes of Veronal buffer by carrying over 0.025 ml volumes of the antigens in calibrated spiral loops. Following addition of 0.025 ml of complement (containing 1.8 full units), the plates were incubated overnight at 4°C, after which the hemolytic system (0.05 ml of 1% sensitized sheep red blood cells) was added. The whole

* This work was supported in part by the Nat. Inst. of Health, Nat. Inst. of Allergy and Infect. Diseases (Contract PH-43-62-200).

† Hyland Laboratories, Los Angeles, Calif.

‡ Cooke Engineering, Alexandria, Va.