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Propagation of Junin Virus, the Etiological Agent of Argentinian Hemorrhagic Fever, in HeLa Cell Cultures. (26728)

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During the late fall and early winter of 1958, an epidemic outbreak of a hemorrhagic, febrile illness occurred in the province of Buenos Aires, Argentine Republic. From the blood of several patients, strains of presumably one virus were isolated by Parodi *et al.*(1) and Pirosky *et al.*(2). Studies by these workers showed that the virus, which has been named Junin, was pathogenic for guinea pigs, newborn mice, and chick embryos. Their reported observations, however, as well as work in these laboratories, indicate that serial propagation of Junin virus in these species is not without difficulties. The incubation period in 1-day-old mice is from 10 to 15 or more days; furthermore, numerous animals survive inoculation at various serial dilutions of virus, with resulting irregular titrations.

Other workers(3) have also reported failure of Junin virus multiplication in monkey kidney tissue cultures and KB and HeLa cells. Nevertheless, it was decided to attempt propagation of the virus in HeLa cells according to methods that have been successfully applied in these laboratories to the study of arthropod-borne viruses(4,5).

Materials and methods. Virus: The present studies were done with strain *XJ* of Junin virus, supplied by Dr. Parodi. Similar investigations with strains *R5* and *F*, supplied by Dr. Pirosky, have yielded preliminary results closely resembling those obtained with strain *XJ*.

Immune sera. Immune sera against Junin virus were produced in mice and rabbits. Mice were given 3 injections of a 10% suspension of infected newborn mouse brain, the first 2 injections intracerebrally at a 3-week interval and the 3rd intraperitoneally; mice were bled 8-15 days after the last injection. Rabbits were inoculated intravenously with 0.5 ml of a 10% suspension of infected mouse brain in physiological saline once a week for 3 consecutive weeks, and exsanguinated 1 month after the last inoculation. Immune sera against other viruses were prepared in the same manner to serve as controls.

Tissue cultures. The stable cell line, strain HeLa (Gey), used throughout was obtained from The Tuskegee Institute in 1954 and has been transferred regularly, first at Division of Laboratories and Research, New York State Dept. of Health, Albany, and since 1957 in these laboratories.

The cells were grown in 160-ml French square bottles in a growth-promoting medium consisting of 45% Hanks' balanced salt solu-

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tion, 15% tryptose phosphate broth (Difco), 30% pooled human adult serum, and 10% fetal bovine serum, supplemented with 100 units of penicillin, 100 μg of streptomycin, and 25 μg of mycostatin per ml. The pH of the Hanks' balanced salt solution was 7.2 to 7.4 and that of the growth-promoting medium about 7.4. The cell inoculum per stock bottle was 10 ml of a cell suspension containing 50,000 cells per ml. The growth-promoting medium was added in amounts of 10 ml per bottle and was changed twice weekly; cellular transfers were carried out regularly at weekly intervals. For cultures of the slant tube type (150 $\times$ 16 mm), trypsin-dispersed cells, 200,000 cells per ml, were dispensed into each tube. In carrying out the cell count, an estimate was made of the number of nuclei in occasional cell clumps. After an initial period of 48-72 hours' incubation at 37°C, monolayers of vigorously growing cells were present and ready to be inoculated.

Inoculation of tubes was carried out as follows: the growth-promoting medium was poured off, leaving behind about 0.2 ml of fluid to which was added 0.7 ml of maintenance medium and 0.1 ml of virus inoculum. The diluent for the latter consisted of 0.75% bovine plasma albumin in phosphate-buffered saline at pH 7.2. The maintenance medium was made up of 97% Eagle's medium(6) and 3% fetal bovine serum supplemented with the same amounts of antibiotics as used in the growth-promoting medium. The pH of the maintenance medium varied between 6.2 and 6.4 and was adjusted with TRIS (Tri(hydroxymethyl)aminomethane), an organic carbon dioxide buffer which has been reported capable of lowering pCO_2 *in vivo*(7) as well as *in vitro*(8). TRIS was prepared as a 2 *M* solution in demineralized water, filtered through a Seitz pad, and added in amounts of 0.3 ml to 100 ml of the maintenance medium so as to maintain the pH of inoculated cell cultures at 7.2 $\pm$ 0.2 during the period of viral synthesis; however, the amount necessary to obtain a physiologic hydrogen-ion concentration varied somewhat.

The pH of the growth-promoting and maintenance media was measured with a Beckman glass electrode pH meter, and that of inoculated cultures was determined colorimetrically, using phenol red in the medium. Cell cultures were examined at intervals of 2 to 3 days and fluids were changed 3 times a week, occasionally daily. The cell cultures were observed for 21 to 28 days after the beginning of the experiment.

Cell cultures were grown on coverglasses placed in Leighton tubes and treated as described(9) for examination by the fluorescent antibody method(10) and by tinctorial methods.

Fluorescent antibody technic†: The serum from a rabbit immunized with Junin virus and a horse antirabbit globulin labeled with fluorescein isothiocyanate adsorbed twice with 100 mg of mouse liver powder on the day of use, were employed in these studies. HeLa cell cultures inoculated with the 5th-passage virus were harvested 7 days later, washed, fixed with acetone for 10 minutes at room temperature, and kept at 4°C until treated with the serum; uninoculated cell cultures were similarly prepared. Both inoculated and uninoculated cells were treated simultaneously, and duplicate specimens were used for all dilutions of serum employed.

The acetone-fixed cells on the coverslips were treated for 30 minutes at room temperature with the Junin virus antiserum at dilution 1:2 in phosphate-buffered saline, pH 7.6. The cells were next washed for 10 minutes with several changes of phosphate-buffered saline, pH 7.4, and treated for 30 minutes at room temperature with the labeled globulin at dilutions 1:2, 1:4, and 1:8. The coverslips were washed as before and the excess fluid removed. The coverslips were then mounted on slides in phosphate-buffered glycerol at pH 7 and examined under the fluorescence microscope on the same day.

Results. Cytopathogenic changes induced by the virus. Strain *XJ* in the 30th newborn mouse passage was inoculated into cell cul-

† The immunofluorescence microscopy was kindly carried out by Dr. Fred Rapp, Philip D. Wilson Research Foundation, The Hospital for Special Surgery, New York; results of fluorescent antibody and tinctorial studies will be published in detail elsewhere.

TABLE I. Serial Passage of Virus Strain *XJ* in HeLa Cells.

No. of passage	Cumulative No. of days in tissue culture	TCD_{50} ($-\log_{10}$/ml)	Cumulative $\log_{10}$ of dilution of original inoculum*
1	16	5.7	5
3	51	3.5	13
5	71	4.5	17
7	96	4.0	21
9	119	5.0	24
10	132	4.5	25

* Original inoculum was a 10% suspension of infected mouse brain.

tures as 10^{-1} to 10^{-5} dilutions of infected mouse brain. Within 5 to 15 days, characteristic changes were observed in the cell cultures. A 1:100 dilution produced cytopathogenic changes by the 5th day in each of the 3 cultures inoculated. Examination of the unstained cell cultures under low magnification (×125) revealed small discrete focal areas of shrunken and granulated cells within the sheet-like outgrowth of cells (Fig. 1, 2). Later, the affected cells tended to fall off the tube wall, with gradual formation of macroscopically recognizable holes. At the end of the 3rd week the whole monolayer was involved, only small islands of normal cells remaining.

The *XJ* strain did not require adaptation to HeLa cells, and over a 4-month period 10 passages have been made in cell cultures with the use of 0.1 ml of either diluted fluid phase or combined cell and fluid phase. This has resulted in dilution of the original inoculum to 10^{-25} (Table I), a value far exceeding the LD_{50} of the source newborn mouse brain suspension. Transfers from infected to new cultures were carried out at 7- to 14-day intervals. Estimation of the virus at the 5th-passage level gave a TCD_{50} titer of $10^{4.5}$ per ml of combined fluid and cell phase. It appears that slightly higher TCD_{50} titers can be obtained by combining the cells and the fluid phase rather than titrating the fluid phase alone.

Examination of coverglass preparations inoculated with 5th-passage material and stained with Giemsa revealed significant cytoplasmic changes in cells localized within the focal lesions. In such cells, polymorphic basophilic bodies of varying size occurred either near the nuclear membrane or irregularly throughout the cytoplasm (Fig. 3).

In fluorescent antibody studies, uninoculated cultures exhibited a diffuse green background, but observations at higher magnification (×400) failed to reveal any localization of antigen in individual cells. In sharp contrast, specific localization of antigen occurred in the infected cultures treated with globulin at dilutions 1:2 and 1:4. Staining was very weak when the labeled reagent was diluted 1:8. Specific staining consisted of antigen aggregates of various sizes in the cytoplasm (Fig. 4). Intranuclear localization of antigen was not evident, and nucleoli showed up very clearly as pale white bodies. The cytoplasmic granules varied considerably in size, from very small bodies to rather large, brightly-staining units (Fig. 4). In large areas of the sheet, the cells did not show specific cytoplasmic fluorescence; cells with fluorescent staining occurred mainly in foci, occasionally as single cells.

Neutralization of the agent propagated in cell cultures by specific immune sera. Additional evidence that the agent propagated *in vitro* was Junin virus, was obtained from neutralization tests. Neutralization of cytopathogenicity has been observed with both mouse and rabbit immune sera. In one test, immune and control rabbit sera were inactivated at 56°C for 30 minutes. Equal amounts of increasing 10-fold dilutions of virus (10% suspension of 30th passage newborn mouse brain) and undiluted serum were mixed and incubated at 37°C for 1 hour. One-tenth ml of the serum-virus mixtures was inoculated into each of 3 HeLa cell cultures per virus dilution. Cell cultures were examined daily and discarded 21 days after the beginning of the test. Results showed that the undiluted serum from a rabbit immunized with Junin virus neutralized 3 log units of the cytopathogenic agent, which reached a titer of 2.5 TCD_{50} ($-\log_{10}$ per ml). The titer in the control tubes with normal rabbit serum was 5.5.

In an additional experiment, virus from the 8th passage in HeLa cells was used. Immune and control rabbit sera were inacti-

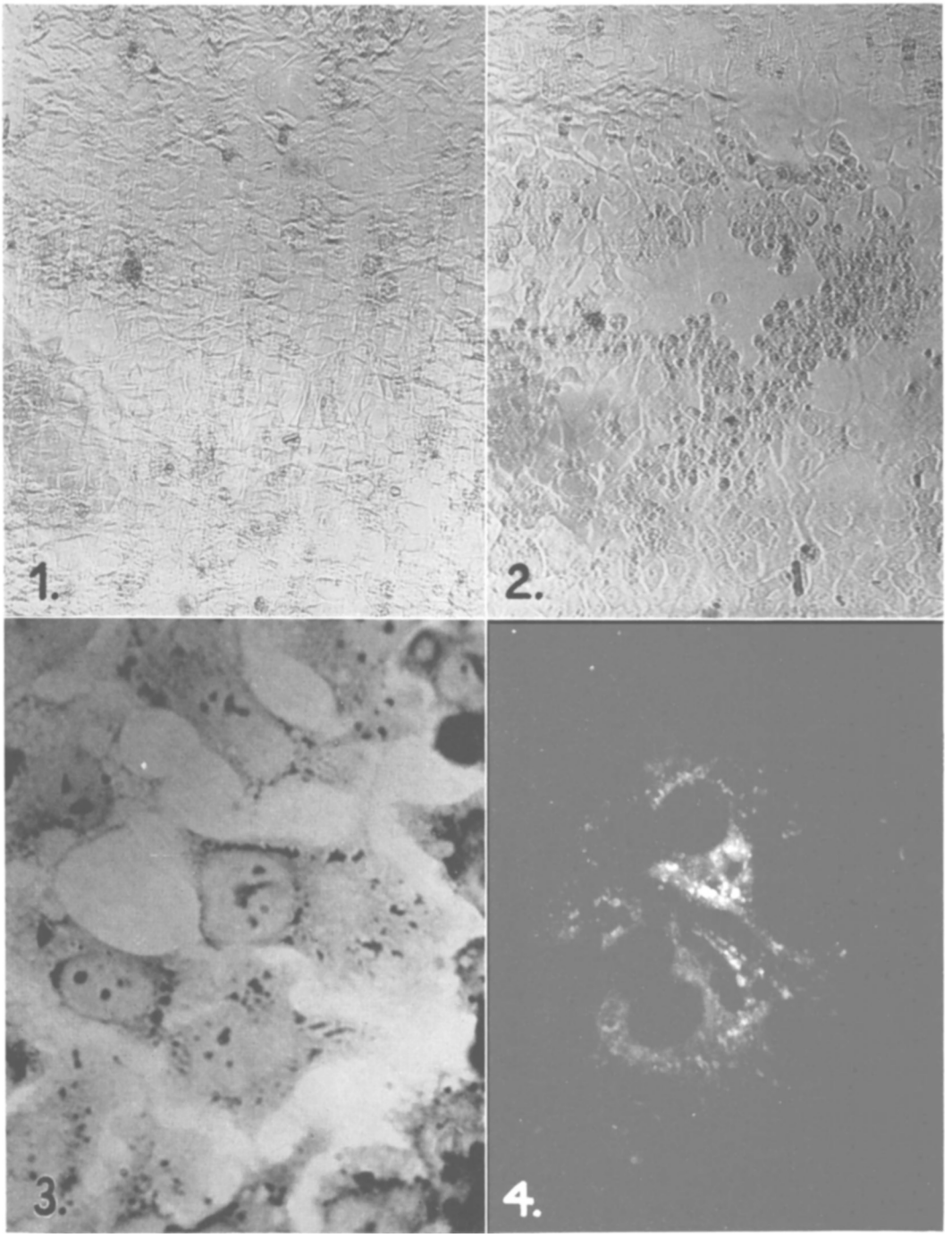

FIG. 1. Uninoculated HeLa cells (×108) at 11 days.

FIG. 2. HeLa cells (×108) inoculated with 2nd passage strain *XJ* virus. Area of focal lesion 11 days after inoculation.

FIG. 3. HeLa cells (×1080), Giemsa stained, 7th day after inoculation with 6th passage strain *XJ* virus. Note basophilic cytoplasmic inclusions.

FIG. 4. Fluorescent antibody preparation of HeLa cells (×1080), 7th day after inoculation with 6th passage strain *XJ* virus. Note specific localization of antigen in cytoplasm.

vated as before. Equal amounts of a 1:10 dilution of combined cell and fluid phase were mixed with increasing 2-fold dilutions of serum, incubated for 1 hour at 37 C, and inoculated into cell cultures. Within 15 days, a 1:40 dilution of the serum from a rabbit immunized with Junin virus neutralized 30 TCD_{50} doses as determined by parallel titration of the virus.

It has been observed that the TCD_{50} titers of Junin virus propagated in 1-day-old mice and titrated in HeLa cells exceed by an average of 1 to 2 log units the TCD_{50} titers of the virus serially maintained in cell cultures and similarly titrated. Under the circumstances, it would seem advisable to use infected mouse brain as source of virus for neutralization tests in which increasing virus dilutions are employed, but to use virus from HeLa cell cultures in the constant virus—varying serum technic and for fluorescent antibody studies.

Effect of sodium desoxycholate on Junin virus. Prior to this study, attempts to determine whether Junin virus was inactivated by sodium desoxycholate had been inconclusive, owing to the irregular outcome of inoculation of serial dilutions of virus in mice. Since sensitivity to this chemical is a property of arthropod-borne viruses(11), it was decided to make use of HeLa cell cultures to obtain definite evidence.

To 1 part of a 10% suspension of mouse brain infected with Junin virus was added 1 part of a 1% solution of sodium desoxycholate. Two control mixtures were prepared, with virus and diluent instead of sodium desoxycholate and with the chemical plus diluent instead of virus. After incubation for 1 hr at 37°C, all mixtures were diluted by increasing 10-fold dilutions to 10^{-7}; 0.1 ml of each dilution was inoculated into each of 2 cell cultures which were examined daily for 15 days. In a similar experiment, virus from the 2nd passage in HeLa cells was used. In the absence of sodium desoxycholate, the virus propagated in mice and in cell cultures had titers (TCD_{50} per ml) of $10^{-4.5}$ and $10^{-4.0}$, respectively; in the presence of sodium desoxycholate, the same virus lines failed to multiply. Rounding and granulation of cells was present in the tubes inoculated with the undiluted mixtures; that this was due to a toxic action of the chemical on the cells and not to virus multiplication was indicated by the fact that control cell cultures inoculated with 1% sodium desoxycholate showed identical changes.

Summary. Junin virus, the etiological agent of Argentinian hemorrhagic fever has been propagated serially in HeLa cell cultures. Appropriate cell viability during the long experimental period was obtained by the use of a suitable combination of growth-promoting and maintenance media as well as by frequent fluid changes during the period of viral synthesis.

A characteristic cytopathic effect was observed during the 1st passage and did not change during 10 consecutive transfers of the agent. Evidence that the cytopathogenic agent was Junin virus was derived from fluorescent antibody studies and from neutralization tests with sera from animals immunized with the virus propagated in newborn mice. In cell cultures, Junin virus was inactivated by sodium desoxycholate, a property common to all arthropod-borne viruses.

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