STUDIES WITH THE ETIOLOGIC AGENT OF ARGENTINIAN EPIDEMIC HEMORRHAGIC FEVER (JUNÍN VIRUS)

FRED RAPP, PH.D., AND SONJA M. BUCKLEY, M.D.

From The Hospital for Special Surgery, Philip D. Wilson Research Foundation, affiliated with The New York Hospital-Cornell Medical Center, the Department of Microbiology and Immunology, Cornell Medical College, and The Rockefeller Foundation Virus Laboratories, New York, N.Y.

In 1955, Arribalzaga[1] described the clinical aspects of a new disease of unknown etiology that had appeared in the northwestern part of the Province of Buenos Aires, Argentina. The recurrence of this hemorrhagic febrile illness in Junín in 1958 enabled investigators to undertake special studies which provided evidence that the causative agent was an arthropod-borne virus, subsequently named Junín virus.[2-4] Other investigators[5] have confirmed these observations. Some properties of the agent were investigated by Greenway and his associates,[6] who reported that certain small laboratory animals, such as guinea pigs and newborn mice and rats, were susceptible to the virus; they were unable, however, to cultivate the virus *in vitro*.

Procedures for the propagation of Junín virus in HeLa (Gey) cells have recently been reported by Mettler, Buckley and Casals.[7] These authors found that inoculation of HeLa cells with 1,000 TCD_{50} of Junín virus, strain XJ,[4] regularly produced a cytopathic effect (CPE) within 5 days. This effect was first manifest as discrete focal lesions composed of shrunken and granulated cells. With extension and coalescence of the foci during the second and third weeks, the cell sheet acquired a "moth-eaten" appearance observable up to 2 months post-inoculation. It was also found that CPE was completely neutralized by serum containing antibody against Junín virus, so that the necessity for using the more cumbersome newborn mouse neutralization test was eliminated.

In view of the potential value of these observations in the diagnosis of Argentinian epidemic hemorrhagic fever, it seemed desirable to investigate the HeLa-Junín virus system in more detail, especially with regard to alterations that occurred in the infected cells prior to the fifth day after inoculation. These further studies were carried out with the parallel use of two methods: the immunologically specific fluorescent

This investigation was supported in part by PHS research grant number E-3823 from the National Institute of Allergy and Infectious Diseases, and in part by grant number C-4372 from the National Cancer Institute, National Institutes of Health, United States Public Health Service.

Accepted for publication, August 11, 1961.

antibody technique,[8] and the conventional nonspecific tinctorial method, the Giemsa stain.

Materials and Methods

Virus

Strain XJ of Junín virus was employed. It had undergone 7 passages in HeLa cells with a resulting dilution of the original inoculum to 10^{-21}. This far exceeded the LD_{50} of $10^{-4.5}$ of the newborn mouse brain suspension that had originally been inoculated into the cell cultures.

Tissue Cultures

Trypsinized HeLa cells were cultured as previously described.[7] Cell suspensions containing 5×10^4 cells per ml. were dispensed in 1 ml. amounts into Leighton tubes containing cover glasses. Monolayers were usually obtained on the cover glasses within 3 days. At this time the growth-promoting fluid[7] was replaced with 0.7 ml. of maintenance solution.[7] The cells were then inoculated with 0.1 ml. of the virus suspension in appropriate dilutions. Titrations of extracellular virus were made in tubes of cells originally seeded with 2×10^5 cells per ml. Fluid from 10 inoculated cell cultures was harvested at appropriate intervals and re-inoculated into fresh cells. The cultures were observed for CPE for 60 days.

Immunofluorescent Method

Immune serum against the virus was prepared in young adult male rabbits by intravenous injection of 0.5 ml. of a 10 per cent suspension of infective mouse brain in 0.85 per cent sodium chloride once a week for 3 consecutive weeks. The rabbits were exsanguinated one month after the last injection. Serums thus prepared have been shown to neutralize the effects of Junín virus in HeLa cells.[7]

The indirect method[9] of the fluorescent antibody technique was employed. Tissue cultures were harvested at suitable intervals post-inoculation, washed once with phosphate buffered saline (pH 7.6), treated with acetone for 10 minutes at room temperature and air-dried; they could then be refrigerated at 4° C. up to 7 days prior to staining. After treatment with normal or immune rabbit serum diluted 1:2 with phosphate-buffered saline (pH 7.6) for 30 minutes at room temperature, the cells were washed for 10 minutes with several changes of phosphate-buffered saline (pH 7.6) and then exposed for 30 minutes to a 1:4 dilution of anti-rabbit globulin horse pseudoglobulin labeled with fluorescein isothiocyanate.[10,11] The fluorescent reagent had previously been absorbed twice with 100 mg. of acetone-precipitated mouse liver powder per ml. to reduce nonspecific staining.[8,12] The cells were re-washed with phosphate-buffered saline for 10 minutes, mounted in phosphate-buffered glycerol (pH 7) and examined with the fluorescent microscope. Controls consisted of (a) uninfected cells reacted with immune rabbit serum and the labeled horse anti-rabbit globulin, and (b) infected cells reacted with the pre-immunization rabbit serum and the labeled horse anti-rabbit globulin.

Giemsa Method

Cell cultures were harvested as above, washed in phosphate-buffered saline, fixed in Bouin's fluid for 15 minutes and allowed to stand overnight in 70 per cent ethyl alcohol. They were then stained in an inverted position with dilute Giemsa stain* (1 ml. of stock solution to 39 ml. of Sorensen's phosphate buffer, pH 7.2) for a total of 72 hours, with changes of stain at 24-hour intervals. The preparations were differentiated in 95 per cent ethyl alcohol containing a few drops of 10 per cent

* Chroma, formerly Grübler and Company, Biological Stains, imported and distributed by Roboz Surgical Instrument Company, Washington 7, D.C.

colophonium alcohol, successively dehydrated in acetone, acetone-xylene (1:1) and xylene, and mounted.

RESULTS

A series of 8 experiments was performed in which cultures of HeLa cells were inoculated with approximately 10,000 TCD_{50} of Junín virus. The sheets were examined for CPE with the low power lens (× 125) of a light microscope; corresponding cover glass cultures were harvested at intervals and stained with Giemsa stain or with the fluorescent antibody reagents. Cultures remained free of visible CPE for two days following inoculation of the cells.

Giemsa Method

Preparations stained with the Giemsa stain and examined at a magnification of 200 times revealed an intact cell sheet consisting of cells with a fine, granular, blue-staining cytoplasm; these cells were occasionally vacuolated (Fig. 1). The nuclei were round, oval, or lobulated and were stained a homogeneous pale blue; they contained 1 to 6 nucleoli which varied considerably in shape and size and stained a dark blue. These cells were similar to those observed in uninfected cultures. Infected cultures at this time also exhibited distinct foci composed of cells containing basophilic cytoplasmic inclusion bodies (Figs. 2 and 3). The number of cells in these foci varied from 1 to approximately 30 but generally averaged between 6 and 10. The inclusion bodies, when observed at higher magnification (× 500) varied in shape; round, oval, rectangular, triangular, threadlike and crescent-like forms were seen. In size they ranged from small granules to bodies having a diameter corresponding to that of larger nucleoli. They were generally located in a juxta-nuclear position, in the ectoplasm, or were found throughout the cytoplasm. Some of the larger bodies had a purple core surrounded by a deeply staining basophilic rim. At still higher magnification (× 800), they appeared as solid masses or were sometimes made up of tiny granules which could be easily distinguished as separate entities. The bodies were frequently surrounded by a clear halo (Fig. 4). Foci of infected cells were invariably surrounded by healthy cells which were similar in every respect to the cells of the uninoculated control cultures.

At this time there was little evidence of cellular destruction, although occasionally a pyknotic cell with basophilic inclusions (Fig. 5) could be seen contracting from the surrounding cells, and a few cells had lysed or exhibited vacuolar degeneration (Fig. 6). In such cells, the nuclei were slightly shrunken but clearly visible and were connected to the cell membrane by threadlike structures which contained basophilic inclusion bodies (Fig. 6).

CPE was observed in unstained infected cultures 3 days after inoculation. This effect consisted of discrete focal lesions characterized by shrunken and granular cells; some of the cells had fallen off the glass, leaving empty spaces within the cell sheet. Giemsa-stained preparations revealed foci that were individually larger (i.e., consisting of at least 30 cells) and also contained basophilic cytoplasmic inclusions that appeared larger than those seen two days post-inoculation. Clear spaces within focal lesions suggested that cells had detached from the glass.

The infection progressed during the following 24 hours. Focal lesions contained up to 100 cells, but the foci were becoming confluent. Cytoplasmic inclusions were often larger than nucleoli. Although stained as before, more bodies were seen in which the peripheral area was more basophilic than the center; occasionally, the whole aggregate stained only faintly. Nuclear alterations were not striking although occasional giant cells were observed.

The destruction of the cell sheet progressed rapidly and was very marked 7 days after inoculation. Stained preparations were unimpressive because many of the infected cells had disappeared, but islands of unaffected cells were still present between infected foci. Giant cells within the lesions were more frequent than in the earlier specimens.

Immunofluorescent Method

Replicate cultures were harvested sequentially at times corresponding to those previously described and were treated with the fluorescent antibody reagents. Uninoculated control cultures were included with each set; these contained no virus antigen as measured by the techniques employed.

Preparations harvested two days after inoculation included a moderate number of single cells containing small, round, cytoplasmic inclusions (Fig. 7). These stained green with the immunofluorescent reagents; they were therefore presumed to contain virus antigen. In addition, groups of cells containing particulate virus antigen were observed (Fig. 8). The antigen-containing bodies varied in size and shape like those seen in the Giemsa-stained preparations (Figs. 2 and 3). Single cells, as well as small foci comprising a number of cells, contained virus antigen scattered throughout the cytoplasm in a diffuse manner (Fig. 9). Such foci, surrounded by cells in which virus antigen could not be detected, seemed to indicate a spread of the infection from an initially infected cell to neighboring cells. Virus antigen was not observed in the nuclei although an occasional body suggested it was present there. It is entirely likely that these inclusions were localized in the cytoplasmic layer above the nucleus since the few cells exhibiting possible

intranuclear inclusions had a large number of inclusions in the cytoplasm.

The infection progressed markedly during the next 24 hours. Foci of infected cells were larger (Fig. 10), and there were more individual cells containing virus antigen (Fig. 11). There was a notable similarity of cells and inclusion bodies shown by the two techniques employed (Figs 5 and 11). In addition, individual inclusions appeared to be larger and brighter. Inclusions appeared to be concentrated near the nuclear membrane or at the edge of the cell. This process continued through the fourth post-inoculation day.

By the seventh day, marked destruction had occurred. Cells had taken on bizarre shapes, and the intensity of the stain for virus antigen had diminished (Fig. 12). In many of the bodies containing antigen, this was concentrated at the periphery while the center was devoid of immunologic reactivity (Fig. 13). This effect, although noted earlier, was now a prominent feature of the infected cells. The appearance thus corresponded to that in the Giemsa-stained preparations in which a basophilic rim surrounded the more faintly staining core of many inclusion bodies.

Persistence of Virus in Tissue Culture Fluid

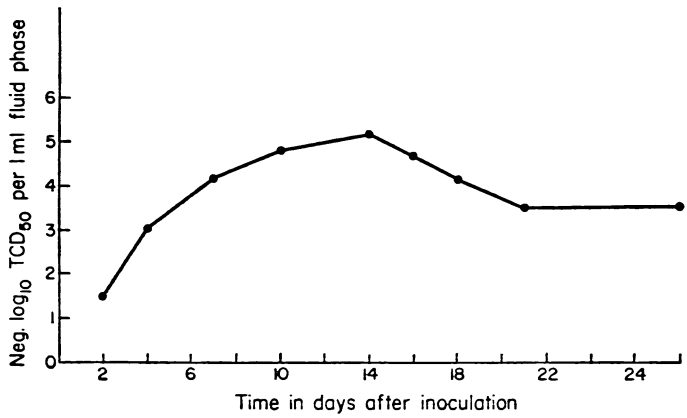
Demonstration of viral effects as early as two days following infection of HeLa cells prompted a preliminary study of the release of infectious virus into the extracellular fluid. Cultures were therefore inoculated with 1,000 TCD_{50} doses of virus and the fluids titrated at intervals following inoculation. Virus was present in the fluid as early as 2 days after inoculation, reached a peak value at 14 days and then decreased (Text-fig. 1). Partial regeneration of the cells occurred 21 days following inoculation. Cell cultures remained chronically infected, and infectious virus was detected in the fluid for as long as 60 days in amounts up to 4,000 TCD_{50} per ml.

Discussion

The reaction of cells to infection with viruses and the localization of virus components can be studied by a variety of techniques. Microscopic examination, both with the light and electron microscope, can be fortified by conventional staining procedures as well as by specific immunologic reactions. The investigation reported here was designed to study further the replication of Junín virus and the reaction of HeLa cells to infection by the simultaneous use of a conventional nonspecific tinctorial method, the Giemsa stain, and the immunologically specific fluorescent antibody method.

In their studies of the HeLa-Junín virus system, Mettler, Buckley

and Casals[7] found that infected cells harvested 7 days after inoculation of virus contained polymorphic, basophilic, cytoplasmic inclusion bodies and that the virus antigens appeared to localize in the cytoplasm of infected cells. Results of the present study, which was directed toward



TEXT-FIGURE 1. Growth curve of Junín virus, strain XJ, of Argentinian epidemic hemorrhagic fever in HeLa cells.

detection of events that occurred earlier in the infected cells, showed that virus antigen could be specifically demonstrated and localized as soon as 48 hours after inoculation of cells when the fluorescent antibody method was used. Even with ordinary light microscopy, CPE caused by strain XJ of Junín virus, and by strains F and R_5 (unpublished results), was visible after another 24 hours, or 3 days after inoculation. From a number of observations, it appeared that the spread of the infection during this 24-hour interval could be extracellular as well as from cell to neighboring cell, although the latter has yet to be conclusively demonstrated. Inhibition of the spread of virus through the fluid phase must be accomplished by use of antibody or with a semisolid overlay.

The basophilic cytoplasmic inclusion bodies seen in the Giemsa-stained preparations appeared to fit Pinkerton's criteria.[13] He suggested: "The visualization of relatively minute aggregates of elementary bodies by the electron microscope probably makes it necessary to broaden our definition of the term inclusion body. Conventional inclusion bodies, however, as seen under the light microscope, range in diameter from 1 or 2 to 20 or more μ in greatest diameter." The bodies observed fit, therefore, into the class of the conventional inclusion body. Study of immunofluorescent preparations suggested that many of the basophilic staining bodies contained virus antigen, a possibility that was supported by the close correlation between the tinctorial and the im-

munofluorescent methods. Proof that all of the basophilic aggregates were composed of virus antigen was not, however, the purpose of this study.

As judged from the different structures observed and the different distribution of antigen in infected cells, ranging from particulate localization to a diffuse distribution, formation of Junín virus in HeLa cells is a dynamic process. The repeated observation of bodies devoid of antigen and of basophilia in the central portion suggested either a virus "scar" or the formation of virus antigen around the periphery of a vacuole. Vacuoles were very prominent in the cells and have been observed in many other types of cells as well. Morgan, Howe and Rose [14] found evidence that another member of the arthropod-borne virus group, Western equine encephalomyelitis virus, was often formed at the periphery of vacuoles in a variety of cells in culture. They postulated that precursor particles differentiated at template sites close to membranes bordering cytoplasmic vacuoles and that these particles either passed into the lumen of the vacuole or dispersed in the cytoplasm and were extruded through the cell wall. They further pointed out that necrosis and dissolution of the cell might occur during this process but that virus could be released without destruction of the cell. Our findings strongly suggested that the Junín virus-HeLa cell system had properties that were similar to their experimental models. The fact that cultures were chronically infected for long periods of time further supported the concept that these cells might survive infection. Similar observations on related viruses have been made by other investigators.[15–17]

Although the fluorescent antibody method is a technique with great usefulness, it must be remembered that the demonstration of inclusion bodies in tissue sections and smears from various organs by conventional methods has been used as a diagnostic procedure for decades.[18] As the present study indicates, one of the advantages to be derived from the combined application of tinctorial and immunofluorescent methods is the conclusive demonstration that changes seen in diseased tissues are related to the production of viral components and are not due to a coexisting secondary disease or an artifact.

The fluorescent antibody technique may well be an important aid in the diagnosis of Argentinian epidemic hemorrhagic fever. At present, the serologic procedure most commonly employed is complement fixation, since neutralization tests, performed either in newborn mice or *in vitro,* are cumbersome and time consuming. Thus, in the absence of a hemagglutination-inhibition test, detection of virus by immunofluorescence may be the most effective laboratory diagnostic aid for this disease.

Summary

The development of Junín virus in HeLa cells has been investigated by the parallel use of a conventional method, the Giemsa stain, and the fluorescent antibody technique. Virus antigen and basophilic inclusion bodies were detected in the cytoplasm of infected cells as early as 48 hours after inoculation. Development of virus antigen was accompanied by a spread of the infection and a release of the virus into the extracellular fluid. Cytopathic effects appeared 3 days following inoculation of the cells and resulted in extensive destruction within 7 days after infection. Chronic infection of the cell cultures was observed for up to 60 days.

The methods employed were used to identify early alterations in the infected cells and to localize specific virus components. This demonstration suggested that the system studied was a useful one for the identification of virus isolates from individuals with Argentinian epidemic hemorrhagic fever.

References

1. Arribalzaga, R. A. Una nueva enfermedad epidémica a germen desconocido. Hipertemia nefrotóxica, leucopénica y enantemática. *Día méd.*, 1955, **27**, 1204–1210.
2. Parodi, A. S.; Greenway, D. J.; Rugiero, H. R.; Frigerio, M.; de la Barrera, J.M.; Mettler, N.; Garzon, F.; Boxaca, M.; de Guerrero, L., and Nota, N. Sobre la etiología del brote epidémico de Junín. *Día méd.*, 1958, **30**, 2300–2301.
3. Greenway, D. J.; Rugiero, H. R., and Parodi, A. S. Fiebre hemorrágica epidémica: sobre un brote en el noroeste de la provincia de Buenos Aires. *Día méd.*, 1959, **31**, 229.
4. Parodi, A. S.; Greenway, D. J.; Rugiero, H. R.; Rivero, E.; Frigerio, M. J.; Mettler, N.; Garzon, F.; Boxaca, M.; de Guerrero, L., and Nota, N. La etiología de la fiebre hemorrágica epidémica de la pcia. de Buenos Aires. *Día méd.*, 1959, **31**, 249–250.
5. Comision Nacional ad Hoc. Virosis hemorrágica del noroeste bonaerense. La primera inoculación experimental al hombre. *Orientación méd.*, 1959, **8**, 144.
6. Greenway, D. J.; Rugiero, H. R.; Parodi, A. S.; Frigerio, M.; Rivero, E., de la Barrera, J. M.; Garzon, F.; Boxaca, M.; Mettler, N.; de Guerrero, L. B.; and Nota, N. Epidemic hemorrhagic fever in Argentina. *Pub. Health Rep.*, 1959, **74**, 1011–1014.
7. Mettler, N.; Buckley, S. M., and Casals, J. Propagation of the etiological agent of Argentinian hemorrhagic fever—Junín virus—in HeLa cell cultures. *Proc. Soc. Exper. Biol. & Med.*, 1961, **107**, 684–688.
8. Coons, A. H., and Kaplan, M. H. Localization of antigen in tissue cells. II. Improvements in a method for the detection of antigen by means of fluorescent antibody. *J. Exper. Med.*, 1950, **91**, 1–13.
9. Weller, T. H., and Coons, A. H. Fluorescent antibody studies with agents of varicella and herpes zoster propagated *in vitro*. *Proc. Soc. Exper. Biol. & Med.*, 1954, **86**, 789–794.

10. RIGGS, J. L.; SEIWALD, R. J.; BURCKHALTER, J. H.; DOWNS, C. M., and METCALF, T. G. Isothiocyanate compounds as fluorescent labeling agents for immune serum. *Am. J. Path.*, 1958, **34**, 1081–1097.

11. MARSHALL, J. D.; EVELAND, W. C., and SMITH, C. W. Superiority of fluorescein isothiocyanate (Riggs) for fluorescent-antibody technic with a modification of its application. *Proc. Soc. Exper. Biol. & Med.*, 1958, **98**, 898–900.

12. COONS, A. H.; LEDUC, E. H., and CONNOLLY, J. M. Studies on antibody production. I. A method for the histochemical demonstration of specific antibody and its application to a study of the hyperimmune rabbit. *J. Exper. Med.*, 1955, **102**, 49–60.

13. PINKERTON, H. Relation of viruses to inclusion bodies. *Ann. New York Acad. Sc.*, 1959, **81**, 207–213.

14. MORGAN, C.; HOWE, C., and ROSE, H. M. Structure and development of viruses as observed in the electron microscope. V. Western equine encephalomyelitis virus. *J. Exper. Med.*, 1961, **113**, 219–234.

15. LOCKHART, R. Z. The production and maintenance of a virus carrier state: the possible role of homologous interference. *Virology*, 1960, **10**, 198–210.

16. DOHERTY, R. L. Effects of yellow fever (17D) and West Nile viruses on the reactions of human appendix and conjunctiva cells to several other viruses. *Virology*, 1958, **6**, 575–583.

17. BANG, F. B.; GEY, G. O.; FOARD, M., and MINNEGAN, D. Chronic infections produced in cultured cell strains by the virus of Eastern equine encephalomyelitis. *Virology*, 1957, **4**, 404–417.

18. NEGRI, A. Beitrag zum Studium der Aetiologie der Tollwut. *Ztschr. Hyg.*, 1903, **43**, 507–527.

[*Illustrations follow*]

LEGENDS FOR FIGURES

Figures 1 to 6 are photomicrographs of HeLa cells inoculated with Junín virus, strain XJ, and stained by the Giemsa method two days after inoculation.

FIG. 1. Uninfected HeLa cells. Vacuoles are evident in apparently "normal" cytoplasm. × 320.

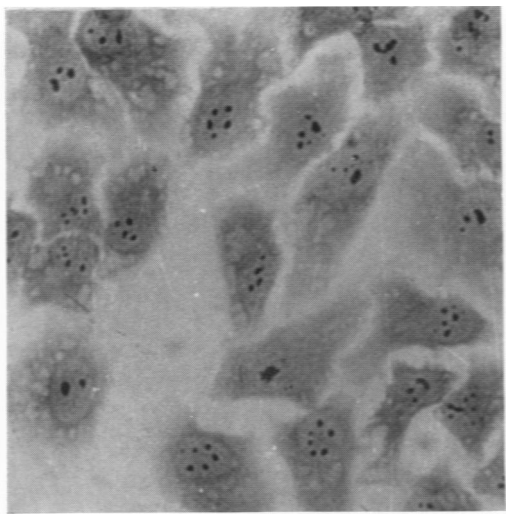
FIG. 2. HeLa cells within a focal lesion. The cell indicated by an arrow contains several basophilic inclusion bodies. × 280.

FIG. 3. A higher magnification of the central area shown in Figure 2. × 1,400.

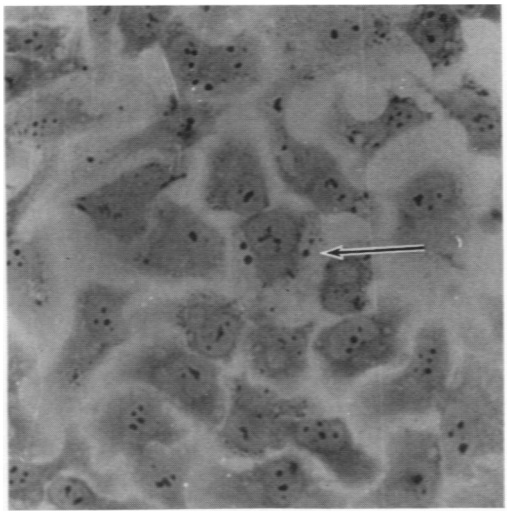
FIG. 4. HeLa cells within a focal lesion. Basophilic cytoplasmic inclusion bodies are surrounded by a halo. × 1,120.

FIG. 5. A pyknotic HeLa cell contains basophilic cytoplasmic inclusion bodies. × 640.

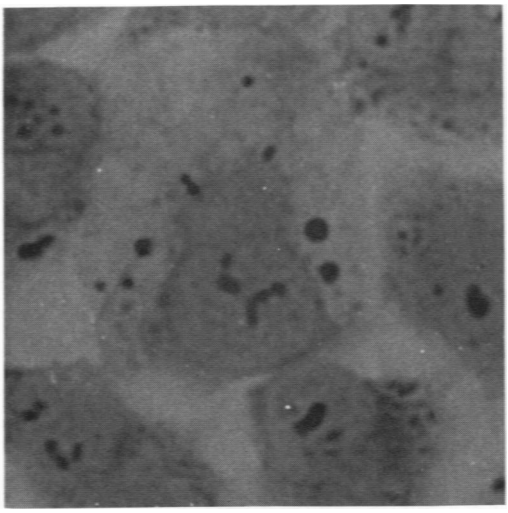
FIG. 6. Vacuolar degeneration is apparent in HeLa cells. × 700.



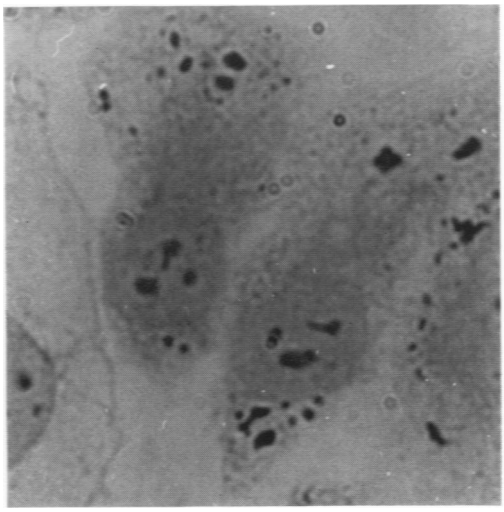
1



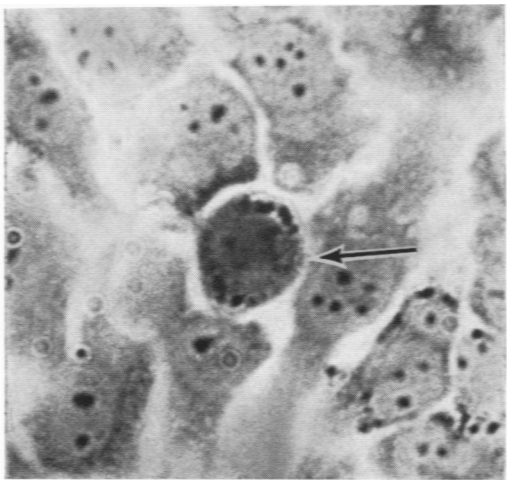
2



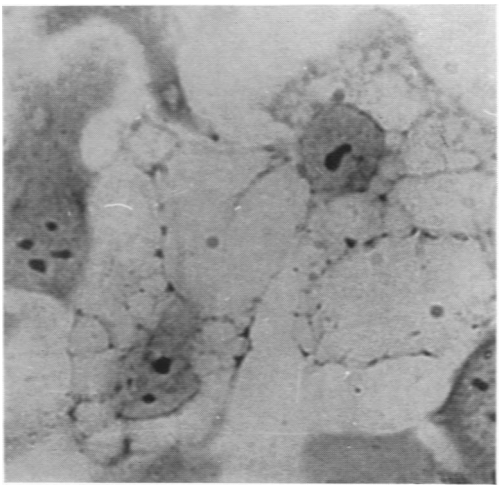
3



4



5



6

FIG. 7. An immunofluorescent micrograph of HeLa cells from a culture infected by Junín virus two days previously. A single infected cell contains small, round, cytoplasmic inclusions; uninfected cells appear in the background. × 750.

FIG. 8. An immunofluorescent micrograph of HeLa cells from a culture infected by Junín virus two days previously. A group of cells contains particulate virus antigen in the cytoplasm. Pale bodies in the dark nuclei are nucleoli. × 750.

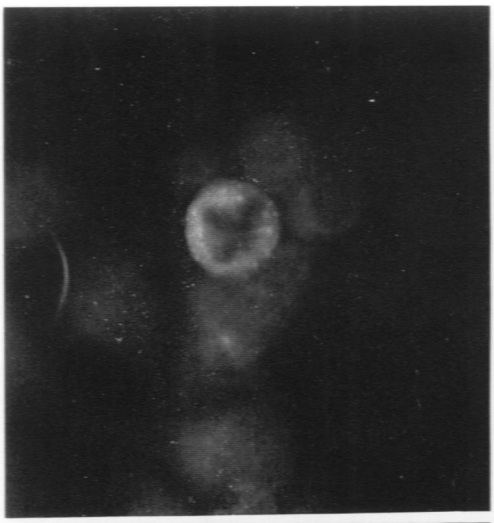
FIG. 9. An immunofluorescent micrograph of HeLa cells from a culture infected by Junín virus two days previously. A focus of cells contains diffuse virus antigen. × 750.

FIG. 10. An immunofluorescent micrograph of HeLa cells from a culture infected by Junín virus 3 days previously. A focus of cells contains particulate virus antigen. There is complete absence of intranuclear staining. × 750.

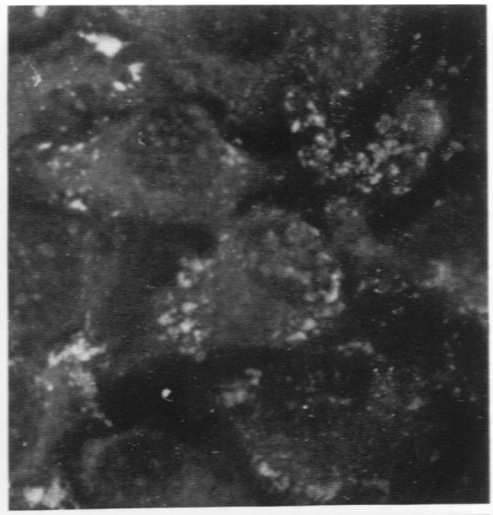
FIG. 11. An immunofluorescent micrograph of HeLa cells from a culture infected by Junín virus 3 days previously. An individual cell contains virus antigen in the vicinity of the cytoplasmic membrane. Compare with Figure 5. × 750.

FIG. 12. An immunofluorescent micrograph of HeLa cells from a culture infected by Junín virus 7 days previously. There is marked cell destruction and a diminution of antigen reaction in many cells. × 750.

FIG. 13. An immunofluorescent micrograph of HeLa cells from a culture infected by Junín virus 7 days previously. Cellular destruction is manifest, and bodies illustrating "empty" core and peripheral virus antigen may be seen. × 750.

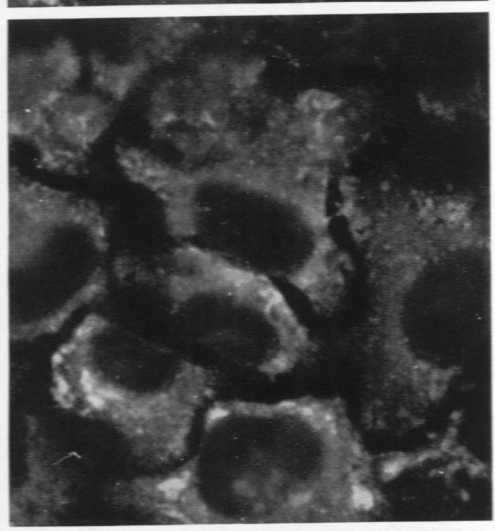


7

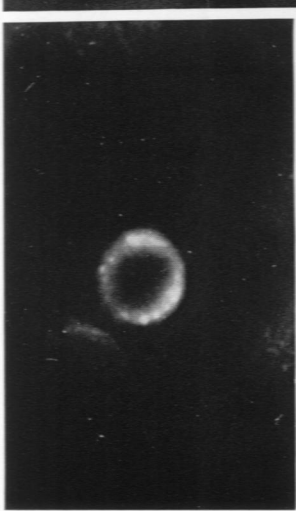


8

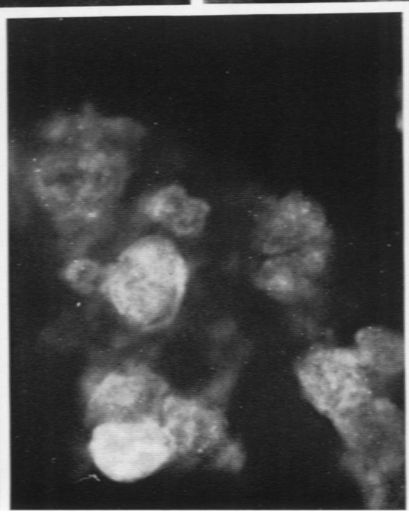
9

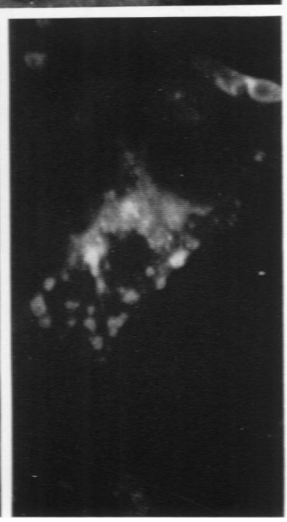


10



11





12, 13