Effect of Hydroxyurea on the Development of African Swine Fever Virus

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VARIOUS STUDIES of the effect of hydroxyurea (HU) on the formation of DNA and its bacteriostatic action have appeared recently.[1-4] The effects on the formation of viral DNA and viral antigens have been reported by Rosenkranz *et al.*,[5] Zambernard,[6] Bell and Maassab,[7] Levy *et al.*,[8] and, most recently, Nii *et al.*[9] In such studies, the suppression of DNA was accompanied by changes in antigen formation that differed somewhat for each virus. The present study of the effect of HU on the morphogenesis of African swine fever virus (ASFV) continues a series of investigations of this virus that have included its development in swine kidney tissue culture cells[10] and the identification of its nucleic acid as DNA.[11,12] The size of ASFV made it particularly suitable for studying the morphologic effects of HU. Furthermore, investigations of HU as an inhibitor of infectivity of this virus might be useful in studies of its immunologic properties—e.g., lack of neutralizing antibody in the presence of complement-fixing and agar gel precipitating antibodies.[13]

Materials and Methods

Virus

The isolate of ASFV, Lisbon-57,[13] in its thirty-sixth to fortieth serial tissue culture passages, was used. The titer was generally $10^{7.5}$ $TCID_{50}$ per milliliter.

Tissue Culture

The PK_{13} cell line* provided virus stocks and virus titration. The culture medium was Earle's salt solution with 0.5% lactalbumin hydrolysate, 0.1% yeast extract, 0.1% bovine albumin, and 5% bovine serum. The amounts of 100 units of penicillin and 100 μg. each of streptomycin and kanamycin were added per milliliter of medium.

Testing Hydroxyurea

Sixteen 4-oz. prescription bottles containing confluent layers of PK_{13} cells were inoculated with 1 ml. of undiluted tissue culture passage virus, which was permitted

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Accepted for publication Jan. 7, 1969.

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to adsorb for 3 hr. in an incubator at 37° C. After rinsing twice with 5 ml. of warm sterile phosphate-buffered saline, two prescription bottles were used for adsorption determinations and two for electron microscopic study. The remaining 12 prescription bottles each received 10 ml. of tissue culture medium containing known amounts of HU and were reincubated. Fresh medium containing the appropriate amounts of HU was placed on these cultures following the first 24 hr. of incubation. Two bottles each were used at 24, 48, and 72 hr. for infectivity determination and electron microscopy.

Virus Titration

Cell layers were prepared for infectivity titration as follows. After they were drained, 2 ml. of normal culture fluid was added to each prescription bottle. The cells were removed by scraping and were transferred quantitatively to a 15-ml. TenBroeck tissue grinder. After they were ground, the disrupted cells were transferred to a 12-ml. conical centrifuge tube, diluted to 10 ml. with culture fluid, and centrifuged for 10 min. at 800 *g*. The sediment volume was used to calculate the dilution factor. Tenfold serial dilutions of the supernatant fluid were made and 1 ml. of each was added to each of two prescription bottles. After absorption for 1 hr. at 37° C., tissue culture fluid was added, and the cultures examined daily for cytopathic changes. The medium was changed twice a week. Titer was calculated by the method of Reed and Muench.[14]

Complement Fixation Test

A modified Kolmer and Boerner complement fixation (CF) test was used.[15] The antigen was tissue culture medium containing 10^{-2} M HU, obtained at specific time intervals, while control antigens were both infected and normal cultures without HU. All fluids were clarified by centrifugation at 800 *g* for 10 min. Degrees of fixation were scored visually from 0–4, with 4 indicating complete fixation. Known positive serum was obtained from swine inoculated with partially attenuated viruses that were refractory to challenge inoculation.

Hydroxyurea

The various concentrations of HU were made up in culture medium with HU from Nutritional Biochemicals Corp.

Electron Microscopy

Cells were scraped from the bottles, collected in pellets, fixed in 1% glutaraldehyde in phosphate buffer for 30 min., and washed and fixed in 2% osmium for 20 min. Following dehydration in graded alcohols, the cells were embedded in epoxy resin (Epon 812). Thin sections were stained with uranyl acetate and examined in an RCA-EMU-3G microscope.

Glycol Methacrylate Embedding and Enzyme Digestion

The methods and enzymes described previously were used with ASFV grown in tissue cultures containing HU.[12]

Results

The biologic and morphologic effects of HU on ASFV at the selected concentrations were examined. Electron micrographs showed increasing

concentrations of particles devoid of central cores as the concentration of HU increased. The morphologic changes were accompanied by a sharp reduction in infectivity, but there was little change in antigenic activity in the fluids as measured by complement fixation.

Figure 1 shows a typical virus assembly site in the cytoplasm 48 hr. after infection with ASFV. The virus particles with darkly stained central cores and a multilayered outer shell are completely assembled ASFV. Immediately adjacent, other virus particles are partially formed and the central core has not yet appeared. This central DNA core becomes visible after the hexagonal shell is formed.[12] A majority of particles (in infected cultures without drug treatment) have outer shells and definite central cores.

At a concentration of 10^{-4} M HU, virus formation is unaffected. Figures 2 and 3 show sites of virus formation in cells treated with 10^{-3} and 10^{-2} M HU, respectively. In these sections, the formation of the outer shell appears to proceed to completion and only a very few particles are seen with central cores. Small structures and portions of outer shell, which precede complete shell formation, are visible in Fig. 3. Cells treated with 10^{-1} M HU were able to replicate virus, but assembly sites were scarce and virus formation was more limited than at lower concentrations (Fig. 4).

The normal formation of ASFV shows virus budding out from the surface of the cell (Fig. 5), and some of the particles have a central core.[10] In HU-treated cultures (Fig. 6), the virus is released from the cell, but there is no indication of a central core in most of the particles. Virus without DNA cores appears in the intercellular spaces.

When HU-treated cultures were embedded in glycol methacrylate and exposed to nuclease digestion, the virus particles rarely were affected. Figure 7 was selected to show that, while most particles are unaffected, two particles in this field had their cores removed. These were particles that presumably contained DNA and were digested by enzyme. The other viruses, in some instances, have indications of core material, but this is probably an artifact of the methacrylate embedding. In the previous study, DNase digestion removed the cores of most particles.[12]

Biologic experiments showed that HU very rapidly reduces the infectivity of the ASFV released into, and titrated in, the fluids of tissue cultures. As shown in Table 1, the effects increased as the concentration of HU was increased. However, even at 10^{-1} M HU, the highest concentration tested, virus production was not suppressed completely. The HU had no toxic effect on the cells. The most consistently useful concentration, 10^{-2} M, reduced the titer of virus in the fluids by four logs at 72

Table 1. Effect of Hydroxyurea on Infectivity of ASFV in Tissue Culture

Sample	Titer* at various hydroxyurea molar concentrations						
	Untreated, no HU	10^{-3}	5×10^{-3}	10^{-2}	10^{-2}	10^{-2}	10^{-1}
Inoculum virus	7.2	7.4	7.3	7.5	7.5	7.5	7.5
3-hr. fluid	6.0	6.5	6.5	5.5	—	—	6.5
cells†	6.0	6.0	7.0	5.0	—	—	4.0
24-hr. fluid	5.5	4.5	4.5	4.0	—	—	6.0
cells	6.5	7.0	6.5	5.0	6.5	6.5	4.0
48-hr. fluid	6.5	5.0	4.5	3.5	3.5	4.5	6.5
cells	7.5	7.5	7.5	4.5	—	—	4.0
72-hr. fluid	6.5	6.0	5.0	4.0	4.0	3.5	6.0
cells	7.5	7.5	7.5	5.0	—	—	—
96-hr. fluid	7.0	—	—	—	—	4.0	—

* Titer expressed at $\log_{10}$ $TCID_{50}$ per milliliter.
† Only those cells which remained attached to the bottles were recovered. The lysed cells and debris were collected as fluid when the bottles were drained.

hr. At the same time, the complement-fixing titer, which was used as a measure of viral and nonstructural antigen (i.e., incomplete virus, damaged or disrupted virus, and antigens derived from viral metabolism), was essentially unchanged (Table 2).

Discussion

The responses in virus formation to the action of HU are not the same for all viruses. Zambernard [6] reported that suppression of viral DNA was not accompanied by complete suppression of viral capsid formation in the frog virus (FV3). High titer T (nonstructural) viral antigens are found when the formation of structural antigen is suppressed by using HU in adenorvirus-infected cells.[8] With influenza virus, hemagglutinin is suppressed by using HU, while infectivity is not affected.[7] The reactions of herpes simplex virus to treatment with HU [9] are similar to those of ASFV. With herpes simplex, empty capsids (devoid of DNA) are formed, but viral envelopes are not assembled. The process with herpes is more complex than with ASFV because of intranuclear particle formation. No nuclear involvement has been observed with ASFV.[10]

Table 2. Effect of Hydroxyurea on Complement-Fixation Titer of ASFV in Tissue Culture

Antigen dilution	Titer* of virus control, no HU	Titer* (with 10^{-3} M HU treatment)			Titer* of normal fluid
		48 hr.	72 hr.	96 hr.	
Undiluted	4	4	4	4	0
1:2	4	4	4	4	0
1:4	4	0	0	4	0
1:8	0	0	0	0	0
1:16	0	0	0	0	0

* Positive fixation graded visually on a scale 0-4.

The normal development of ASFV appears to follow a pattern in which the DNA core is formed after assembly of the characteristic hexagonal shell.[10,12] Deoxyribonucleic acid may be transferred into the capsid after virion assembly, but experimental data is not complete on this point. Autoradiographic studies are in progress to examine this transfer.

Experiments will be performed to determine whether inoculation of animals with virus-containing fluids obtained from HU-treated cultures result in immunologic responses different from those observed previously with complete virus. The possible increase in antigenic mass with a lower infectivity may lead to formation of neutralizing antibody that was absent in all other experiments.[12]

Summary

Treating infected culture cells with hydroxyurea inhibited the formation of the central DNA core of African swine fever virus. At the same time, however, the outer structure of the coreless particles appeared as complete as those of fully mature virus particles. The infectivity titer of culture fluids was reduced by approximately four logs with 10^{-2} M hydroxyurea, while the production of complement-fixing antigen was not affected significantly. Coreless virus was present both in the cytoplasm and after budding from the cell. Deoxyribonuclease had no effect on such particles.

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The PK_{15} cell line used for tissue culture was obtained through the courtesy of Dr. S. H. Madin, Naval Biological Laboratory, University of California, Berkeley, Calif.

Legends for Figures

The black lines (magnification indicators) in each of Fig. 1–7 represent 1 μ.

Fig. 1. African swine fever virus particles 48 hr. after infection of PK_{15} cell cultures. Note the definite central cores and the hexagonal outer shell. No HU.

Fig. 2. African swine fever virus assembly area. Complete and incomplete hexagonal shells are visible, as well as earlier stages of viral formation. Two particles have cores. At 48 hr. after infection; 10^{-3} M HU.

Fig. 3. African swine fever virus assembly area. Most particles appear to have no cores. Hexagonal shell is well defined. At 48 hr. after infection; 10^{-3} M HU.

Fig. 4. African swine fever virus assembly area after exposure to 10^{-1} M HU, 48 hr. after infection. Very few virus particles are formed. No central cores visible. Nucleus visible in lower right hand corner.

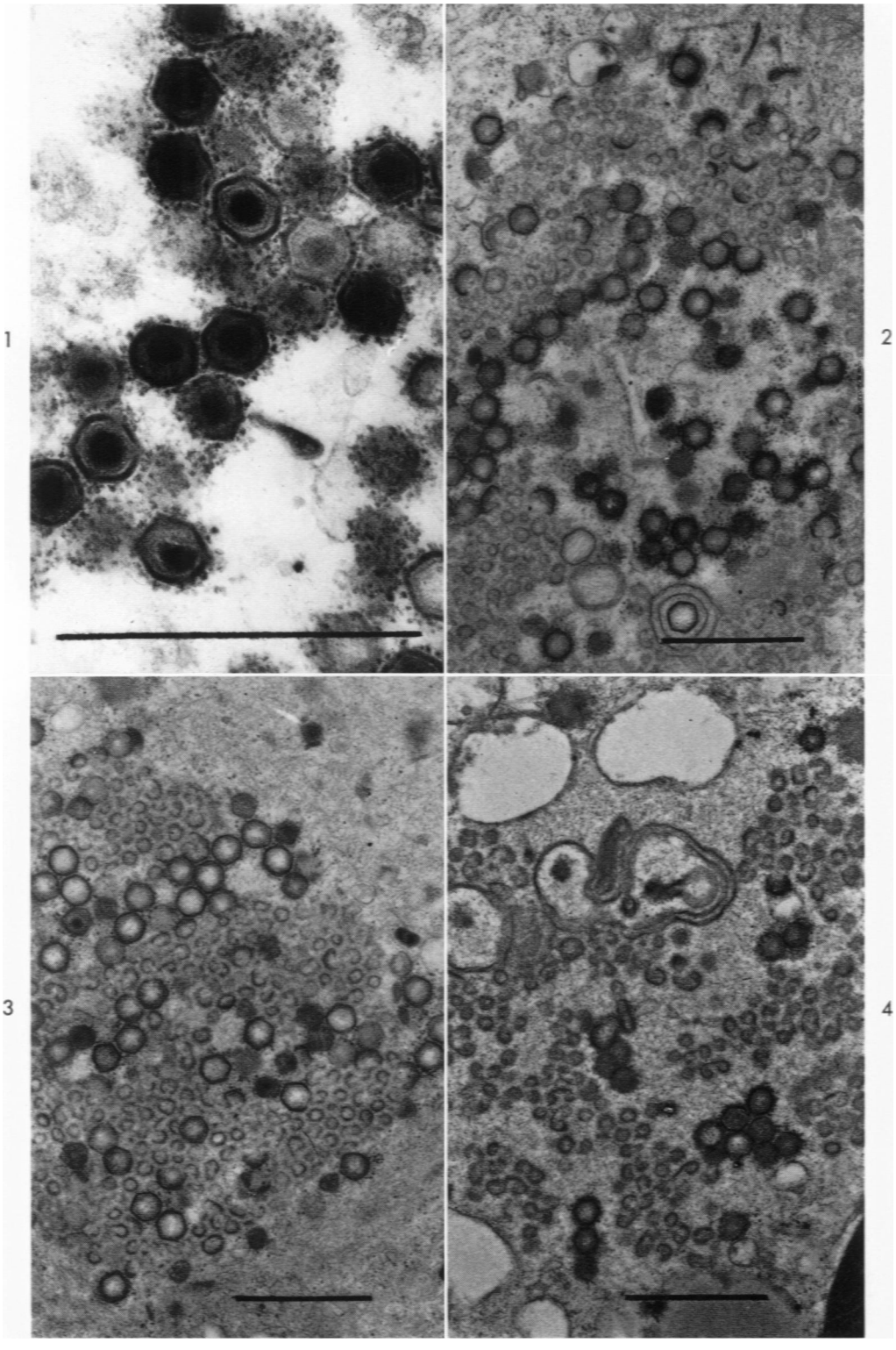
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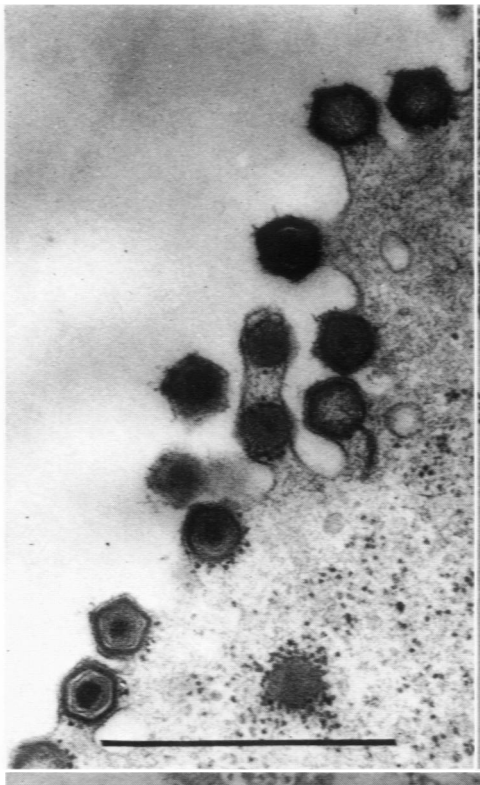
Fig. 5. African swine fever virus budding from surface of PK_{15} culture cell. At 48 hr. after infection; no HU.

Fig. 6. Hydroxyurea-treated African swine fever virus outside cell. Characteristic hexagonal outer shell, but no dense central cores. At 72 hr. after infection; 10^{-2} M HU.

Fig. 7. A large area of virus particles in section embedded in glycol methacrylate and digested with 0.3% DNase for 3 hr. Cells had been infected for 72 hr.; 10^{-2} M HU. Two particles *(arrows)* were digested, indicating active DNase.



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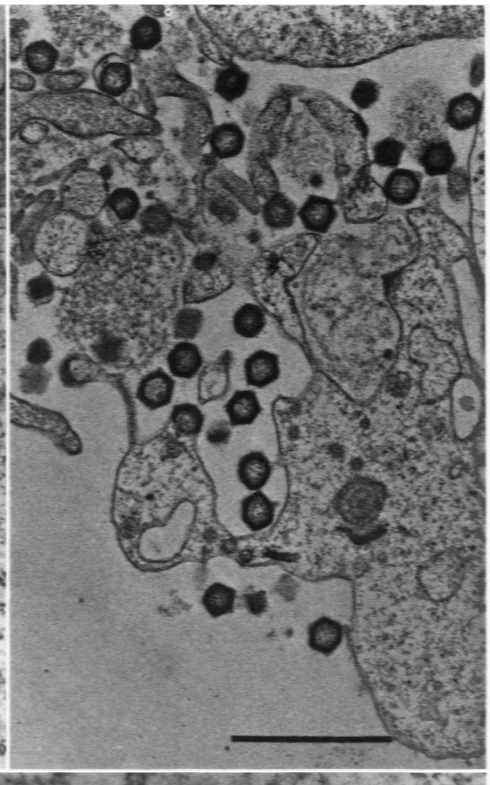

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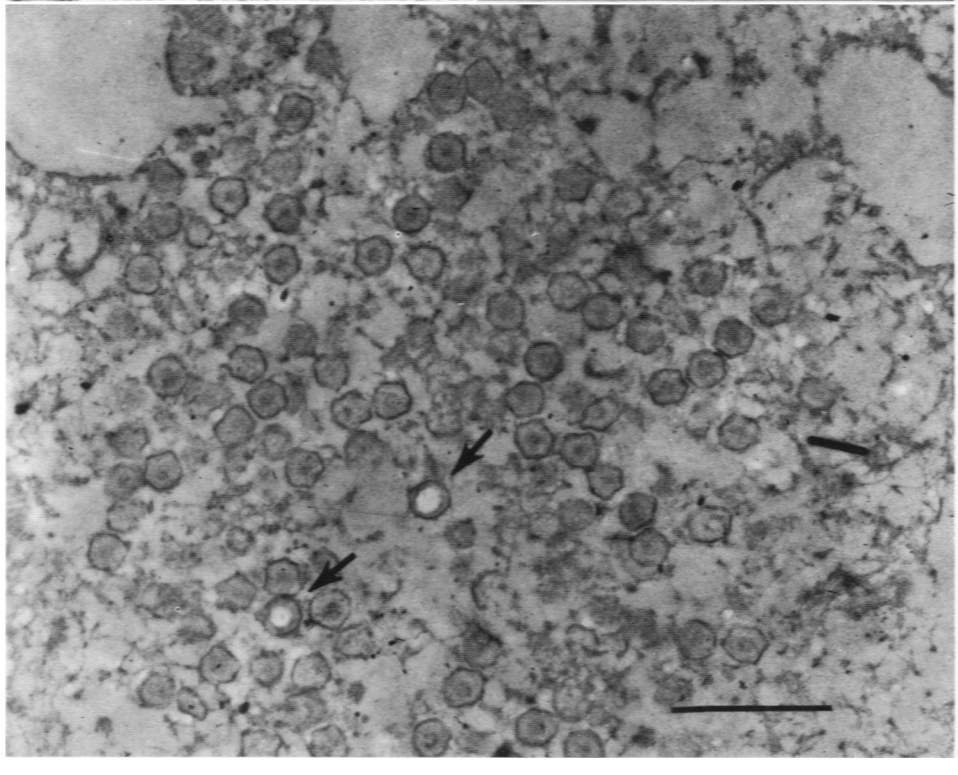

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