Inhibition of Group B Arbovirus Antigen Production and Replication in Cells Enucleated with Cytochalasin B

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A comparative study of the growth of Sindbis (SIN) virus, a group A arbovirus (togavirus), and Japanese encephalitis (JE) virus, a representative group B arbovirus (togavirus), was conducted in enucleate and nucleate cells. Immunofluorescent tests and yield measurements demonstrated that chicken embryo cells which had been enucleated and subsequently infected with SIN virus produced virus-specific antigens and infectious virus. By contrast, JE failed to replicate or produce virus-specific antigen in cells which had been enucleated before or even 2 h post infection. Studies of the effect of enucleation at various times after infection demonstrated that a nucleus must be present at least 2 and possibly as long as 4 h after infection to produce either JE-specific antigen or infectious JE virus. These studies demonstrate that the replication of SIN, a group A arbovirus (togavirus), which has no nuclear requirement, contrasts sharply with that of a group B arbovirus (togavirus), JE, which may have an initial dependence on a nucleus-associated process.

An examination of the replication patterns of group A and group B togaviruses reveals a number of differences between the two groups. A comparative examination of the kinetics of replication has shown that the replication cycle of the group B arboviruses (togaviruses) is considerably longer than those of group A (12). In chicken embryo cells (CEC), Japanese encephalitis (JE) virus, the representative group B arbovirus (togavirus) used in these studies, has a latent period of about 10 h (17), twice as long as that of group A arboviruses (togaviruses) such as Sindbis (SIN) virus (13). This difference suggests that early processes in group B togavirus replication may be of a more complex nature. Little information exists on the intracellular events occurring during the latent period of group B togavirus replication. Ultrastructural studies of infected cells have revealed no morphological changes until the appearance of cytoplasmic virus-filled vesicles during the logarithmic phase of viral replication (8, 9, 11). However, the electron microscope studies of Yasuzumi et al. (22–24) describe viral precursors in the nucleus during the latent period, as well as degenerative nuclear changes during the course of infection. In addition, a few biochemical studies also suggest the possibility of nuclear involvement (19; T. A. Brawner, J. C. Lee, and D. W. Trent, Abstr. Am. Soc. Microbiol. V 48, p. 202, 1973).

Using the recently developed technology for mass enucleation of populations of cells by treatment with cytochalasin B (14), we have inquired into the necessity of a nucleus for the replication of JE. Furthermore, by enucleation at various times subsequent to the time of infection, we can address the question of how long a nucleus must be present for this group B togavirus to replicate. Since there is no indication that group A togaviruses require a nuclear step in their replication, a comparative study using SIN virus has been conducted as a control.

MATERIALS AND METHODS

Cells. Primary CEC prepared from 12-day-old embryos were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum (DME-10% FCS). Secondary cultures were plated onto plastic disks about 23 mm in diameter that had been punched out of the bottom of collagen-coated, plastic tissue culture dishes and sterilized by UV irradiation.

Viruses. Viruses used in this study were: (i) the 27th mouse brain passage of the M1/311 strain (3) of JE virus, and (ii) a suckling mouse brain seed of SIN virus strain AR339 (21).

Enucleation. Cells were enucleated by a modification of the procedure developed by Prescott et al. (14). Plastic disks on which secondary cultures of CEC had been grown were inverted into 50-ml plastic centrifuge tubes which contained 8 ml of DME-10% FCS with 10 µg of cytochalasin B per ml (Aldrich Chemical Co., Milwaukee, Wis.). They were centrifuged at 37 C for

15 min at 12,000 rpm in the SS-34 rotor in a Sorvall RC2B centrifuge. The disks of cells were then washed with a balanced salt solution and placed at 37 C in 35-mm petri dishes containing 2 ml of DME-10% FCS. Recovery was accomplished within 15 to 30 min with >95% enucleation.

After recovery, if the cells were to be used in the immunofluorescence assay, the disks were washed once with Puck saline G (16), a phosphate-buffered saline-containing glucose (GPBS) from which we omitted calcium and magnesium, and placed into small beakers containing 10 ml of 0.25% trypsin (GIBCO) for 5 min at room temperature. Using a rubber policeman, the cells were scraped off into the trypsin, 15 ml of DME-2% FCS was added, and the cells were centrifuged at 2,000 rpm for 10 min. Cells were resuspended in DME-10% FCS and plated into chamber slides (Lab-Tek, Westmont, Ill.). Cells from four disks were used for each chamber slide.

Infection. Cells were infected at a multiplicity of infection of 50 using either JE (M1/311) virus or SIN (AR339) virus with DME-20% FCS as diluent. After an adsorption period of 1 h at 37 C, the cells were washed extensively with GPBS and DME-20% FCS was added.

Immunofluorescence assay. Cells were washed twice with GPBS, fixed for 5 min in absolute methanol, rinsed again with GPBS, and frozen at -70 C until used. Hyperimmune mouse ascitic fluid, prepared against JE or SIN by previously described methods (2), or normal mouse ascitic fluid was placed on the cells and incubated for 30 min at 37 C. The hyperimmune mouse ascitic fluid (or normal mouse ascitic fluid) was drained off and the cells were washed on a shaker at 37 C for 1 h with three changes of 0.02 M phosphate-buffered saline, pH 7.2. Fluorescein-conjugated goat anti-mouse gamma globulin (Antibodies, Inc., Davis, Calif.) was added to each slide and incubated for 30 min at 37 C. The conjugated antiserum was removed and once again the cells were washed on a shaker at 37 C for 1 h with three changes of phosphate-buffered saline. Glass coverslips were mounted on the slides with 90% glycerol 10% phosphate-buffered saline. Slides were then viewed using an American Optical Microstar fluorescent microscope, equipped with a mercury lamp (HBO-200 W), a Corning no. 5840 exciter filter, an opaque glass heat-absorbing filter, and a Schott OG-1 barrier filter. Photographs were taken with a Nikon Dark Box model 35S equipped with a Nikon AFM microflex body, using Kodak Tri-X film ASA 400.

Assay of virus infectivity. Samples of culture fluid from infected cells were diluted and used to infect LLC-MK₂ cells, an established line of monkey kidney cells. After adsorption for 1 h at 37 C, 7 ml of an overlay of the following composition was added to the cells: 1% purified agar (Difco), 10% FCS, 10% (10X) medium 199, 0.3% NaHCO₃, 0.5% (100X) vitamins, 0.5% (100X) BME amino acids, 0.2% DEAE-dextran, 100 U of penicillin, and 100 U of streptomycin. The cells were then incubated for 4 days if infected with SIN or 7 days if infected with JE, at which time 0.5 ml of a 1:1,500 dilution of neutral red (GIBCO) in normal saline was added to each

flask. After incubation at 37 C for 2 h, the cells were left covered at room temperature overnight and plaques were counted the next day.

RESULTS

Comparison of the production of viral antigens in nucleate and enucleate cells. Nucleate and enucleate CEC were infected with either SIN virus or JE virus and were assayed for virus-specific antigens using the indirect fluorescent antibody technique. Cells were enucleated before infection and fixed and examined at 18 h postinfection. The cells were counted and the percentage which showed virus-specific fluorescence was determined. Of the cells infected with SIN virus, 83% of the nucleate cells fluoresced as compared to 81% of enucleate cells. The viral fluorescence was of the same intensity in both types of cultures and involved the entire cytoplasm (Fig. 1, A and B). These results indicated that production of group A togavirus antigens does not require the nucleus.

Repetition of the above experiment with JE virus produced opposite results. No enucleate cells (Fig. 1D) exhibiting JE-specific fluorescence were observed after scanning 1,000 cells, whereas 69% of the nucleate cells showed JE-specific fluorescence. This indicates the requirement for a nucleus-associated function.

Comparison of the production of infectious virus by arbovirus (togavirus)-infected nucleate and enucleate cells. In addition to assaying for the synthesis of viral antigens, cultures of nucleate and enucleate togavirus-infected cells were tested for the production of infectious virus. Samples of infected culture fluid were removed during the latent period and late in the virus replication cycle, i.e., at 2 h and 10 h postinfection in the case of SIN-infected cells (13) and 2 and 24 h postinfection in JE-infected cells (17). The samples were titered by plaque assay in LLC-MK₂ cells and the results are shown in Table 1. In agreement with the immunofluorescent data, SIN-infected enucleate cells produced infectious virus, whereas JE-infected enucleate cells did not. The slight rise in titer in the JE-enucleate cultures may be accounted for by the 1% of nucleate cells remaining after cytochalasin B treatment. Both JE- and SIN-infected cells which had been treated with cytochalasin B but not enucleated gave yields of virus comparable to nucleate-infected cells.

Kinetics of the nuclear phase of JE replication. To determine the temporal relationship of nuclear involvement to JE replication, cells infected with JE virus were enucleated at 2, 4, 6, or 10 h postinfection. All
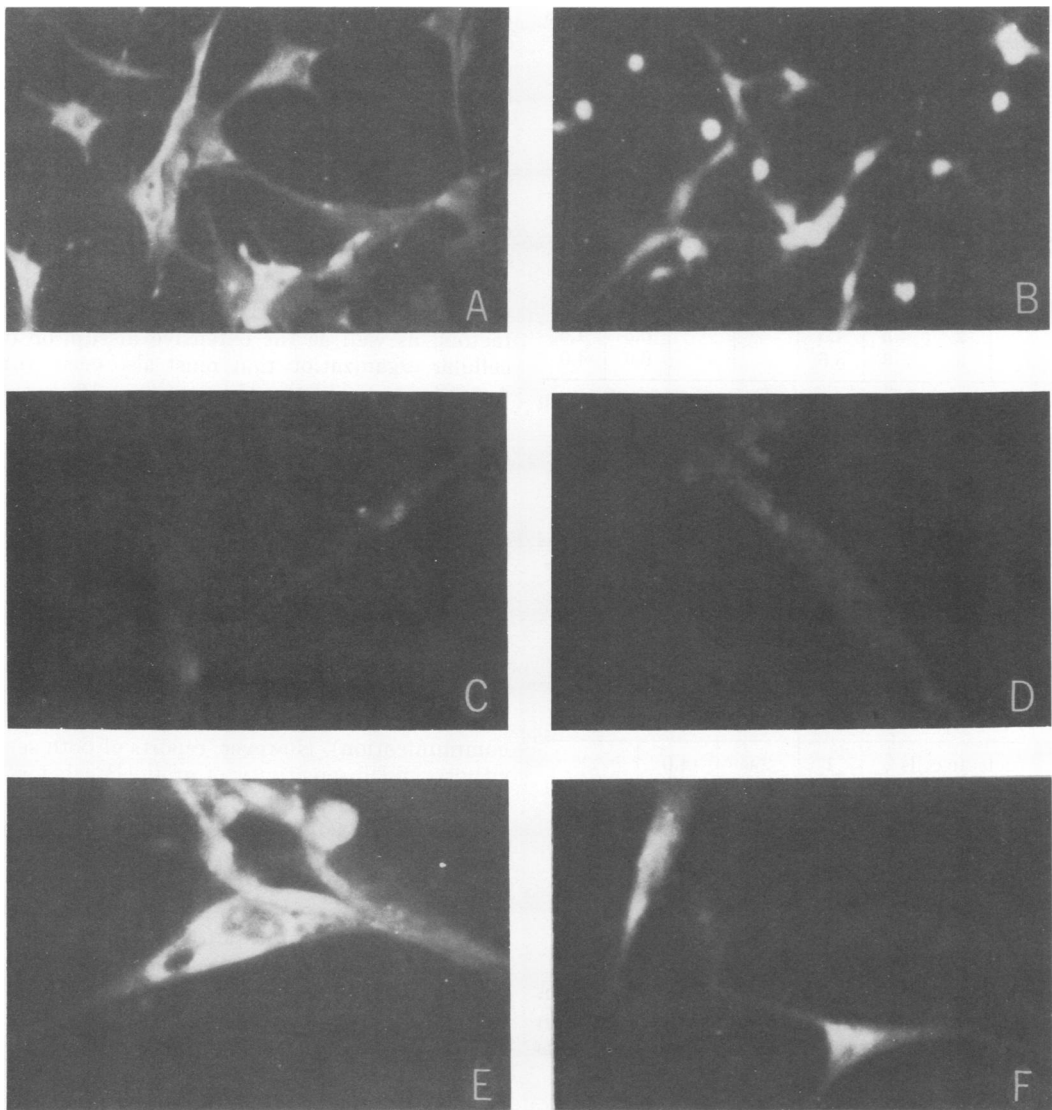
Fig. 1. *Immunofluorescent patterns of CEC observed using specified hyperimmune mouse ascitic fluid (HMAF) and fluorescein-conjugated goat anti-mouse gamma globulin. (A) SIN virus-infected, nucleated CEC, treated with SIN virus-specific HMAF; (B) SIN virus-infected, enucleated CEC, treated with SIN virus-specific HMAF; (C) uninfected, enucleated CEC, treated with JE virus-specific HMAF; (D) JE virus-infected, enucleated CEC, treated with JE virus-specific HMAF; (E) JE virus-infected, nucleated CEC, treated with JE virus-specific HMAF; (F) JE virus-infected CEC, enucleated at 10 h postinfection and treated with JE virus-specific HMAF.*

cultures were assayed for virus-specific immunofluorescence at 18 h postinfection and samples of culture fluid were taken at 10 h (during the latent period) and 24 h postinfection. Results are shown in Table 2. These data indicated that the nucleus-requiring phase occurs during the latent period, being completed in all cells by 10 h postinfection, with a minimum time of 4 h being required.

In the nucleated JE-infected CEC, the fluorescence was confined to the cytoplasm and was most intense in the perinuclear area (Fig. 1E). Infected cells enucleated after the nuclear phase showed fluorescence throughout the cell. Often the most intensely fluorescing region appeared in the center of the cell, the space normally occupied by the nucleus (Fig. 1F). This suggests that the perinuclear area is an

TABLE 1. *Comparison of the production of infectious virus in togavirus-infected nucleate and enucleate chicken embryo cells*

Virus	Expt	PFU/ml of culture fluid ($\log_{10}$)					
		2 h post-infection[a]		10 h post-infection		24 h post-infection	
		N[b]	E	N	E	N	E
SIN	1	4.4	4.3	6.7	5.1		
	2	4.3	4.5	6.1	5.5		
JE	1	4.0	3.8			5.7	4.0
	2	4.3	4.3			6.3	4.6
	3	3.3	3.6			6.0	4.0

[a] An inoculum of 10^8 PFU of virus was used in all cases.

[b] N, nucleate; E, enucleate.

TABLE 2. *Production of infectious virus by JE-infected chicken embryo cells enucleated at various times after infection*

JE-infected chicken embryo cells	Expt	PFU/ml of culture fluid ($\log_{10}$)		% cells showing virus-specific immuno-fluo-rescence
		10 h post-infection	24 h post-infection	
Nucleate cells	1	2.6	4.0	71
	2	2.8	3.8	
Enucleated 4 h postinfection	1	2.8	3.5	03
	2	2.7	3.3	
Enucleated 6 h postinfection	1	2.8	3.5	34
	2	2.6	3.6	
Enucleated 10 h postinfection	1	2.6	4.0	68
	2	2.6	4.0	

important site for viral antigen synthesis, in agreement with immunofluorescent data on cells infected with another group B arbovirus (togavirus), dengue-2 (4).

DISCUSSION

The data presented in this paper indicate a major basic difference in the mechanism of replication of group A and group B togaviruses. Synthesis of viral antigens and production of infectious virus in cells infected with SIN was clearly not dependent on the presence of the cell nucleus, whereas the presence of the nucleus was essential for JE virus replication.

Enucleate cells infected with SIN virus produced infectious virus, thus demonstrating that not only the direction of the synthesis of virus-specific antigens, but also the completion of all of the biosynthetic and morphogenetic steps necessary for the production of infectious progeny virus, proceeds in the absence of a nucleus.

However, the yield of infectious virus from enucleate cells was reduced compared to the nucleate controls. This reduction in yield may be attributable to several factors. Fewer cells were plated in the cultures to be enucleated and some additional cell loss (~10%) occurred during centrifugation. In addition, enucleate cells are considerably smaller than nucleate cells. Since a significant portion of the plasma membrane is lost during enucleation, the total area available for maturation of this budding virus is proportionally reduced. All of the above factors, as well as the extensive disruption of cellular organization that must also occur (6), may be responsible for the decreased efficiency of infectious virus production.

In contrast, it was clear from yield and immunofluorescent studies that the nuclear phase of JE replication in most of the cells of the population was completed by 10 h postinfection. Our data are congruent with observations made by others during studies with metabolic inhibitors. Cordycepin, as well as actinomycin D and daunomycin, prevents the replication of Saint Louis encephalitis, another group B togavirus, in BHK 21/13 cells when added during the first 9 h after infection (D. Trent, personal communication). However, reports of both sensitivity and insensitivity of group B arbovirus (togavirus) replication to actinomycin D exist (10, 17–19, 21, 25, 26). These can be explained on the basis of variations in time of addition and dose of the drug, as well as variation in the response of the host function in different cell types. The fact that conditions can be determined where group B replication is sensitive to inhibitors of nuclear functions does suggest the existence of a nuclear phase of virus replication. In contrast, replication of group A togaviruses is insensitive to actinomycin D under all conditions and, under the appropriate circumstances, the yield of infectious virus is even enhanced (7, 16). The sensitivity of the replication of Saint Louis encephalitis to cordycepin, combined with the existence of a nuclear-associated viral induced 45S RNA with the same sedimentation coefficient as virion RNA but a higher poly (A) content (5.6 to 7.8% versus 2.4% for virion RNA) (Brawner et al., Abstr. Am. Soc. Microbiol. V 28, p. 202, 1973) suggests that post-transcriptional addition using a host-specified nucleus-associated enzyme occurs. Other support for nuclear involvement in group B togavirus replication includes autoradiographic studies, indicating that early in infection viral RNA synthesis occurs in the nucleus (19).

Studies also exist to suggest the involvement

of the perinuclear area in virus replication. Using 1% Triton X-100, Zeibovitz (25) could remove virus-specific RNA from the nuclear fraction of group B togavirus-infected cells without disruption of the nucleus. Hence, Zebovitz's suggestion that the site of viral RNA synthesis is not the nucleus per se, but the outer nuclear membrane, cannot be ruled out and must be considered in formulating a scheme of replication for group B togaviruses. Likewise, immunofluorescent data indicate the involvement of the perinuclear area of the cell in viral antigen production (4). Nuclear fluorescence has never been reported (1, 4, 5). However, all of the above studies were conducted on cells during the logarithmic phase of virus replication and hence do not conflict with a nuclear involvement during the latent period, as we suggest.

One possible interpretation of our data is that some early essential step in the replication of JE, a representative group B togavirus, occurs in the nucleus. The most probable basis for nuclear participation in group B arbovirus replication is a host-mediated process involving modification of the viral genome by post-transcriptional addition of a poly(A) sequence.

ACKNOWLEDGMENTS

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