Sedimentation Changes of L Cells in a Density Gradient Early After Infection with Vaccinia Virus

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By 2 h postinfection, LM cells infected with vaccinia virus show a shift in their distribution when separated on a Ficoll density gradient. This shift is dependent on both time and the multiplicity of infection and is due, at least in part, to an increase in cell size. Those cells which do shift in position in the gradient represent infected members of the population. Physical changes induced in virus-infected cells can be utilized for studying early events in virus replication.

When cultured mammalian cells are infected with animal viruses, an asynchronous infection usually results (3); that is, not every cell is equally susceptible or sensitive to infection by a given virus. A number of factors may contribute to this difference in sensitivity, including the density of suspension cultures, the degree of confluence of monolayer cultures, and the nutritional state of the medium. The underlying basis of all these factors might be the eukaryotic cell cycle which has been shown in some systems to affect virus replication (5, 8, 9).

Results using gradients of Ficoll (7, 13, 14) have shown their great potential for separating a mixture of cells into different cell types; furthermore, Ficoll gradients can separate members of a population of a single type of cell into classes according to the phase of the cell cycle (M. O. Creighton, Ph.D thesis, Univ. of Western Ontario, London, 1971; 1, 14).

Recently, several reports indicated that density changes occur in virus-infected cells. These changes were sufficient to be detectable when the infected cell populations were centrifuged on density gradients. Thus, by 6 days postinfection (PI) Sugawara et al. (15) were able to separate Epstein-Barr virus-infected cells from uninfected cells. Fox and Levine (4) partially separated simian virus 40-induced cells from noninduced cells by 24 h PI. Sykes et al. (16) detected an enrichment of cells in the topmost zone of their gradients at an unspecified length of time after infection with vaccinia virus. With the exception of Fox and Levine's work, a biochemical explanation has not been sought,

and very early changes were not studied, as we shall report here.

The purpose of the present study was to show that it is possible to separate virus-sensitive or "recruited" LM cells from virus-insensitive or "unrecruited" LM cells very early after infection with vaccinia virus by using gradients made from Ficoll and that this separation can be applied to studying early events of virus replication.

MATERIALS AND METHODS

Cells. Conditions for growth of suspension and monolayer cultures of LM cells have been described (11). Except where noted, log-phase suspension cultures were employed to ensure having cells in all stages of the cell cycle.

Virus. Vaccinia virus (10) used for this study was grown in suspension cultures of LM cells. Log-phase cells were centrifuged for 15 min at $1,000 \times g$. Medium 199 plus peptone and methylcellulose (M199P + MC) (11) was removed and the cell pellet was resuspended in sonically treated stock vaccinia virus plus fresh M199P + MC to a cell concentration of approximately 5×10^7 cells/ml. This material was mixed slowly at 35 C for 1 h, after which fresh M199P + MC was added to achieve a final cell concentration of approximately 5×10^8 cells/ml. The infected cell suspension was incubated for 72 h at 35 C in a screw-capped Erlenmeyer flask on a shaker incubator, after which the infected cells were handled as described previously (10). For studies reported here, virus was partially purified by layering twice over 36% sucrose and centrifuging at $46,000 \times g$ for 1 h. Methods for titrating vaccinia virus have been described (10).

Method of infection. To effect maximum adsorp-

tion of virus onto cells, approximately 5×10^7 LM cells were pelleted from suspension and resuspended in 1.0 ml of sonically treated, partially purified vaccinia virus in M199P + MC. Mock-infected cells were treated similarly but resuspended in 1.0 ml of M199P + MC. The mixtures were slowly rotated at 4 C for 1 h in a screw-capped, 7.5-ml siliconized bijou bottle. The cells were then aseptically transferred to a centrifuge tube. The bijou bottle was washed twice with 5-ml samples of medium, which were pooled with the original virus-cell suspension. After centrifugation at $1,000 \times g$ for 15 min at 4 C to remove unadsorbed virus, the cell pellet was resuspended in fresh medium to a concentration of 2×10^8 to 5×10^8 cells/ml and placed in a screw-capped Erlenmeyer flask at 35 C on a rotary shaker. This time was considered to be zero hour postinfection.

Density gradient centrifugation of cells. Density gradients of Ficoll (Pharmacia, Uppsala, Sweden), a high-molecular-weight polymer of sucrose, were used for cell distribution analyses. Ficoll solutions were made in either of the following ways. (i) For nonsterile work a stock solution of Ficoll was prepared at a concentration of 18% (wt/vol), dissolved in M199P by stirring at 4 C, and stored frozen between uses. (ii) For sterile work, 18 g of Ficoll was autoclaved for 15 min at approximately 112 C and then dissolved in sterile M199P to a volume of 100 ml, as above. This material was also stored frozen between uses.

Each gradient consisted of five layers, each 2 ml in volume. The concentrations of Ficoll in the layers were 14, 15, 16, 17, and 18%, in M199P. As each layer was added it was allowed to slowly run down the inside wall of a 16- by 100-mm glass culture tube, thereby forming clearly visible interfaces.

A 2.5-ml amount of an infected or mock-infected cell suspension, at a concentration of 2×10^8 to 5×10^8 cells/ml, was layered on top of each gradient. The gradients were centrifuged in a Sorvall HB4 swinging bucket rotor at $1,000 \times g$ for 15 min at 4 C. After centrifugation the interfaces were still clearly visible. Centrifuged cells formed opalescent bands at these interfaces. The gradients were unloaded from the top by a Buchler Densiflow density gradient remover. Seven-drop fractions (approximately 0.4 ml) were collected by an LKB fraction collector. Samples from each fraction were counted twice on a Coulter counter model A, and the values were averaged.

Infectious center assay. To technically facilitate the aseptic fractionation, larger fraction volumes (1.0 ml) were taken. A sample from each fraction was serially diluted through a series of 10-fold dilutions, and 1.0-ml samples of each dilution were placed onto duplicate preformed confluent LM cell monolayers in Multidish Dispo Trays (Linbro Chemical Co., Inc., New Haven, Conn.). M199P (2 ml) was added to each dish, and the trays were placed without shaking in a 37 C incubator, in an atmosphere of 95% air, 5% CO₂. The trays were left undisturbed for 48 to 72 h and then were fixed and stained for plaque counting (11).

Cell sizing. Samples from unfractionated cultures were appropriately diluted with isotonic saline and analyzed on a Celscope counter linked to a Nuclear Data pulse height channel analyzer and chart re-

corder. Since the data from the counter provided only relative size values, standard particles of known diameter (mulberry pollen, 12–13 μ m, and ragweed pollen, 19–20 μ m) were also analyzed to estimate changes in absolute cell diameter.

RESULTS

Kinetics of the cell distribution shift in a density gradient after infection. A density gradient centrifugation analysis of cells infected with vaccinia virus at a multiplicity of infection (MOI) of 10 was performed at varying times postinfection by using samples from a single suspension culture throughout. A mock-infected culture was analyzed concurrently. By comparing Fig. 1A (0 h PI) with Fig. 1D (3 h PI), it was evident that a significant shift occurred in the cell distribution profile. This shift was time dependent (Fig. 1B, C), reaching its maximum by 2 to 3 h PI. The shift was towards the top of the gradient and represented a displacement of approximately 13 fractions, or one-half the gradient volume. The mock-infected cell culture distribution, up to 3 h PI, remained identical to the profile of the zero-hour vaccinia virus-infected cell culture (Fig. 1A). This experiment was repeated 15 times with similar results. Mock- and virus-infected cells, both before and after centrifugation, were examined for clumping. The number of clumped cells was found to be negligible in all cases.

Effect of varying the MOI on the cell distribution shift. For this experiment four cultures were used; one was mock infected and the other three were infected with vaccinia virus at MOI values of 0.1, 1.0, and 10.0. At 2 h PI, all four cultures were analyzed on Ficoll gradients. The results are shown in Fig. 2. As in the kinetic study, there appeared to be a gradual shift in the profile toward the top of the gradient, in this case as the MOI was increased. We interpreted this to mean that, as more cells became infected (by increasing the MOI), more of them would locate nearer the top of the gradient.

Location of infected cells in a gradient. From Fig. 2 we reasoned that those cells of a culture which actually shifted towards the top of the gradient represented the infected members of the culture. To show this it was necessary to use an MOI less than 1. Under these conditions statistically not every cell would become infected with a virion. We could then ask whether those cells which did shift in position were the virus-infected members of the population. We chose an MOI of 0.1, which at 2 h PI would be predicted to result in a negligible shift in the gradient profile (Fig. 2B). After separation of the mock-infected and virus-

Fig. 1. Kinetics of the shift in cell distribution profile due to infection of LM cells with vaccinia virus, at an MOI of 10. Cells were infected and analyzed on Ficoll density gradients, as described in Materials and Methods. A, Zero hour postinfection; B, 1 h postinfection; C, 2 h postinfection; D, 3 h postinfection. The distribution of mock-infected cells, from zero to 3 h postinfection, was as in panel A.

infected cells on sterile gradients, samples were removed aseptically for cell counts and infectious center assays. The results of this experiment are shown in Fig. 3. The curve representing the results of the infectious center assay ("percent plaque-forming cells per fraction"), which was calculated from the data of the other two curves, showed that cells which located nearer the top of the gradient (e.g., fraction 3), although relatively few in number, contained approximately a fourfold greater number of virus producers (44 versus 11%) than the majority of cells, which located nearer the bottom of the gradient (e.g., fraction 5). This was taken to indicate that those cells of an infected population which shifted towards the top of the gradient represented infected members of the population. In a parallel experiment the virus-infected cell culture was analyzed at both 2 and 8 h PI. It was found that the percentage of virus-producing cells in a pool of fractions 1 to 4 increased from 2 to 8 h PI, with a concomitant decrease in the percentage of virus-producing cells in fraction 5.

Increase in cell size after infection. Earlier work indicated that the basis of separation of cells on Ficoll gradients was the difference in cell size or cell density (2, 12, 13). To establish the basis for our system, we subjected mock-infected and virus-infected cells to electronic cell sizing. Due to small numbers, however, it was technically impossible to size cells from any single fraction of the gradient. Consequently, only entire unfractionated cell populations were analyzed. Using an MOI of 10, cell cultures were analyzed at 3 h PI. The results from this analysis (Fig. 4) showed that cells from the virus-infected culture increased in size relative to cells from the mock-infected culture. In comparison with particles of known diameter, we calculated that the diameters of uninfected cells ranged from 14.4 μm to 26.0 μm , whereas the diameters of virus-infected cells ranged from 14.8 μm to 27.2 μm . These cell diameter changes can be calculated to correspond to mean cell volume changes of 10 to 20%. These data therefore corroborated previous reports.

DISCUSSION

The results we present here show that very early (2 h) after infection of a suspension culture of LM cells with vaccinia virus, a change occurs in the distribution pattern of these cells on a Ficoll density gradient. This change in the distribution profile, which takes the form of a shift towards the top of the gradient, is at least partly due to an increase in the size of the cells

of the infected culture. The shift is both time dependent and MOI dependent.

Under conditions in which not every cell can become infected (MOI less than 1), it appears that those cells which do become infected locate nearer the top of the gradient.

Several recent reports indicating density changes in virus-infected cells may be related to the phenomenon we describe. Sugawara et al. (15) reported that Epstein-Barr (EB) virus-infected P3HR-1 cells, 6 days PI, could be separated from uninfected cells on discontinuous gum acacia gradients. This separation was due to a decrease in the buoyant density of the infected cells as EB viral synthesis proceeded. Sykes et al. (16) reported the separation of vaccinia virus-infected CMP and ME-180 cells from uninfected cells on a discontinuous Ficoll gradient. They observed a twofold increase in the percentage of cells in the topmost zone of the gradient of virus-infected cells compared to control cells but did not indicate at what time postinfection the analysis was carried out. Fox and Levine (4) described the partial separation of simian virus 40-induced 3T3 cells from noninduced cells by sedimentation through linear Ficoll gradients 24 h after infection. This separation was due to an increase in size of the induced cells (in S phase) over the noninduced cells (in G phase).

We feel that the sedimentation changes of infected cells which we describe in this communication may be due to virus-induced cell surface alterations. If this were the case, the alterations might be related or equivalent to those described by Zarling and Tevethia (19). They reported that by 2 h PI of rabbit kidney cells with vaccinia virus, cell surface changes occurred which were manifested as an acquired ability to bind the plant agglutinin concanavalin A. This acquisition occurred early in the virus replicative cycle and preceded new virus synthesis by 4 h. Furthermore, Ueda et al. (17) reported that by 2 h PI of HeLa cells with vaccinia virus, a cell surface antigen could be detected by the direct fluorescent antibody technique. The appearance of this surface antigen could not be prevented by the addition of cytosine arabinoside at a concentration which suppressed the synthesis of cytoplasmic viral antigens.

The following explanation is offered for the phenomenon we describe. Soon after vaccinia

FIG. 2. Effect of the MOI on the cell distribution profile at 2 h PI. Cells were infected and analyzed on Ficoll density gradients, as described in Materials and Methods. A, Mock-infected cells; B, MOI equals 0.1; C, MOI equals 1.0; D, MOI equals 10.0.

FIG. 3. Location of infected cells in a Ficoll gradient at 2 h PI at an MOI of 0.1. Cells were infected and analyzed aseptically on sterile Ficoll gradients, as described in Materials and Methods. Each 1-ml fraction was assayed for cell count (O) and for plaque-forming cells ($\times$). From these data, the percent of plaque-forming cells per fraction ($\bullet$) was calculated.

FIG. 4. Cell-size distribution of mock-infected (—) and virus-infected (----) cells at an MOI of 10, 3 h PI. Each curve was generated by counting at least 10^5 cells until comparable peak heights were obtained. A (arrow), mean diameter ($12.5 \mu\text{m}$) of a suspension of mulberry pollen; B (arrow), mean diameter ($19.5 \mu\text{m}$) of a suspension of ragweed pollen.

virus infects susceptible cells, the virion-bound DNA-dependent RNA polymerase transcribes part of the parental DNA. Heat inactivation causes a denaturation of this enzyme (6, 18), but antibody neutralization presumably does not. We believe that the virion-bound DNA-

dependent RNA polymerase initiates the sequence of events which leads to the sedimentation shift of vaccinia virus-infected cells reported in this communication. In agreement with this hypothesis, we found that by using rabbit antivaccinia virus antibody, in an amount which reduced the titer by more than 99% both in neutralization and infectious center assays, we have not been able to prevent the cell distribution shift from occurring. However, by heating the virus at 56°C for 45 min, which reduced the titer by more than 99%, we succeeded in completely inhibiting the occurrence of this shift.

If our hypothesis is correct, then it should also be possible to prevent the occurrence of the shift by inhibiting macromolecular synthesis (especially RNA and protein) in infected cells. Preliminary results of experiments designed with this purpose in mind indicate that, under conditions in which 99% of incorporation of the respective radioactive precursors into trichloroacetic acid-precipitable material is inhibited, the distribution shift phenomenon occurs in the presence of cytosine arabinoside but does not occur in the presence of cycloheximide. This data would suggest that DNA synthesis is not required for the occurrence of the phenomenon, but that protein synthesis is required. Experiments using actinomycin D are presently in

progress. Experiments are also in progress to see whether the shift phenomenon is correlated with the appearance of early vaccinia virus-induced cell surface changes.

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ADDENDUM

During the revision of this manuscript, Esteban and Metz (J. Gen. Virol. 19:201-216, 1973) reported that virus-specific transcription and translation could be detected within 20 min after infection of L cells by vaccinia virus. This would substantiate our proposal that a very early virus-directed synthetic event could account for our results.

LITERATURE CITED

1. Ayad, S. R., M. Fox, and D. Winstanley. 1969. The use of Ficoll gradient centrifugation to produce synchronous mouse lymphoma cells. *Biochem. Biophys. Res. Commun.* **37**:551-558.
2. Boone, C. W., G. S. Harell, and H. E. Bond. 1968. The resolution of mixtures of viable mammalian cells into homogeneous fractions by zonal centrifugation. *J. Cell Biol.* **36**:369-378.
3. Cairns, J. 1960. The initiation of vaccinia infection. *Virology* **11**:603-623.
4. Fox, T. O., and A. J. Levine. 1971. Relationship between virus-induced cellular deoxyribonucleic acid synthesis and transformation by simian virus 40. *J. Virol.* **7**:473-477.
5. Groyon, R. M., and A. J. Kniazeff. 1967. Vaccinia virus infection of synchronized pig kidney cells. *J. Virol.* **1**:1255-1264.
6. Joklik, W. K. 1968. The poxviruses. *Annu. Rev. Microbiol.* **22**:359-390.
7. Macdonald, H. R., and R. G. Miller. 1970. Synchronization of mouse L-cells by a velocity sedimentation technique. *Biophys. J.* **10**:834-842.
8. Mantani, M., and S. Kato. 1972. Lack of virus-induced cell surface antigen in metaphase-arrest cells infected with poxvirus. *Biken J.* **15**:105-109.
9. Marcus, P. I., and E. Robbins. 1963. Viral inhibition in the metaphase-arrest cell. *Proc. Nat. Acad. Sci. U.S.A.* **50**:1156-1164.
10. Medzon, E. L., and H. Bauer. 1970. Structural features of vaccinia virus revealed by negative staining, sectioning, and freeze-etching. *Virology* **40**:860-867.
11. Medzon, E. L., and D. J. Merchant. 1971. Interaction of the LM cell surface with methylcellulose and vaccinia virus. Mode of action and implications for large scale vaccine production. *In Vitro* **7**:46-58.
12. Miller, R. G., and R. A. Phillips. 1969. Separation of cells by velocity sedimentation. *J. Cell. Physiol.* **73**:191-202.
13. Pretlow, T. G., and C. W. Boone. 1969. Separation of mammalian cells using programmed gradient sedimentation. *Exp. Mol. Pathol.* **11**:139-152.
14. Sinclair, R., and D. H. L. Bishop. 1965. Synchronous culture of strain-L mouse cells. *Nature (London)* **205**:1272-1273.
15. Sugawara, K., F. Mizuno, and T. Osata. 1971. Density changes of cultured Burkitt lymphoma cells following EB viral synthesis. *Nature N. Biol.* **233**:106-107.
16. Sykes, J. A., J. Whitescarver, L. Briggs, and J. H. Anson. 1970. Separation of tumor cells from fibroblasts with use of discontinuous density gradients. *J. Nat. Cancer Inst.* **44**:855-864.
17. Ueda, Y., M. Ito, and I. Tagaya. 1969. A specific surface antigen induced by poxvirus. *Virology* **38**:180-182.
18. Woodson, B. 1968. Recent progress in poxvirus research. *Bacteriol. Rev.* **32**:127-137.
19. Zarling, J., and S. S. Tevethia. 1971. Expression of concanavalin A binding sites in rabbit kidney cells infected with vaccinia virus. *Virology* **45**:313-316.