Metabolism of Lecithin and Virus-Induced Cell Fusion* (33683)

PETER ELSBACH[1], KATHRYN V. HOLMES[2], AND PURNELL W. CHOPPIN

Department of Medicine, New York University School of Medicine, and The Rockefeller University, New York, New York 10021

Cell fusion can be induced by many viruses, particularly the subgroup II myxoviruses and the herpes viruses (1, 2); however, the mechanisms involved in the fusion reaction remain unclear despite the efforts of many investigators using several different viruses. Baby hamster kidney (BHK21-F) cells are extremely sensitive to fusion induced by the simian parainfluenza virus SV5 (3–5). Essentially complete fusion of a monolayer can be induced by concentrated virus within 1–2 hr without virus multiplication, or, with virus replication, within 14–18 hr after infection at a multiplicity of 16 pfu/cell. The massive fusion of cells into a large syncytium clearly indicates that significant changes occur in the plasma membranes of the cells. The characteristics of the cell fusion reaction, including temperature dependence and a pH optimum (3, 6), suggest that there may be an attack by viral and/or cellular enzymes on membrane constituents. Since structural integrity of biological membranes appears to depend in large part on lipid components (7), we examined various aspects of metabolism of a major phospholipid, lecithin, during rapid cell fusion caused by inoculation with concentrated SV5, and during fusion occurring as a result of SV5 infection. The findings indicate that the SV5 virion does not possess detectable phospholipase activity, and that fusion of BHK21-F cells by SV5 is not accompanied by an accelerated breakdown of cellular lecithin or accumulation of lysolecithin.

Materials and Methods. Virus. The W3 strain of SV5 was propagated, concentrated, and partially purified by differential centrifugation as described previously (3, 8), and purified further by equilibrium sedimentation in a potassium tartrate gradient (9).

Cells. The BHK21-F cell line (3) is a heteroploid variant of the BHK21 line isolated by Macpherson and Stoker (10). Monolayer cultures were grown in 60-mm plastic petri dishes and inoculated with SV5 as described previously (3). The medium used during cell fusion experiments was reinforced Eagle's medium (11) with 2% calf serum. Cultures were incubated at 37° in a humidified atmosphere at 5% CO_2 in air.

Biochemical methods. Cellular phospholipids were labeled during preincubation of cells for 22 hr with choline methyl-^{14}C chloride, 40 mCi/mmole (New England Nuclear Corp., Boston, Mass.). Lecithin-^{32}P and lysolecithin-^{32}P were prepared biosynthetically by incubating rat liver slices with $^{32}P_i$, and subsequent isolation and purification as described in detail recently (12–14). The radiochemical purity in different preparations of these labeled substrates ranged from 92 to 98% for lecithin, and from 98.5 to 99.5% for lysolecithin.

The composition of the assay mixtures is described in the figure legends. Incubation was carried out at 37° in a shaking waterbath with room air as the gas phase. Enzymatic activity was stopped by the addition of chloroform:methanol (1:1, v:v) when ^{32}P labeled substrates were used, and with chloroform:methanol (2:1, v:v) when cellular lipids were labeled by preincubation with choline methyl-^{14}C. Lipid extraction was allowed to proceed overnight at room temperature. The chloroform:methanol (2:1, v:v) extracts were washed with 7 m*M* $CaCl_2$ as described by Folch *et al.* (15) to remove ^{14}C-labeled water-soluble substances. All lipid extracts were filtered, dried under reduced pressure at 37°, and applied to silica gel G thin-layer plates. Chromatography, identifica-

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[1] Career scientist of the Health Research Council of the City of New York (Contract I-379).

[2] Present address: The Biological Laboratories, Harvard University, Cambridge, Massachusetts.

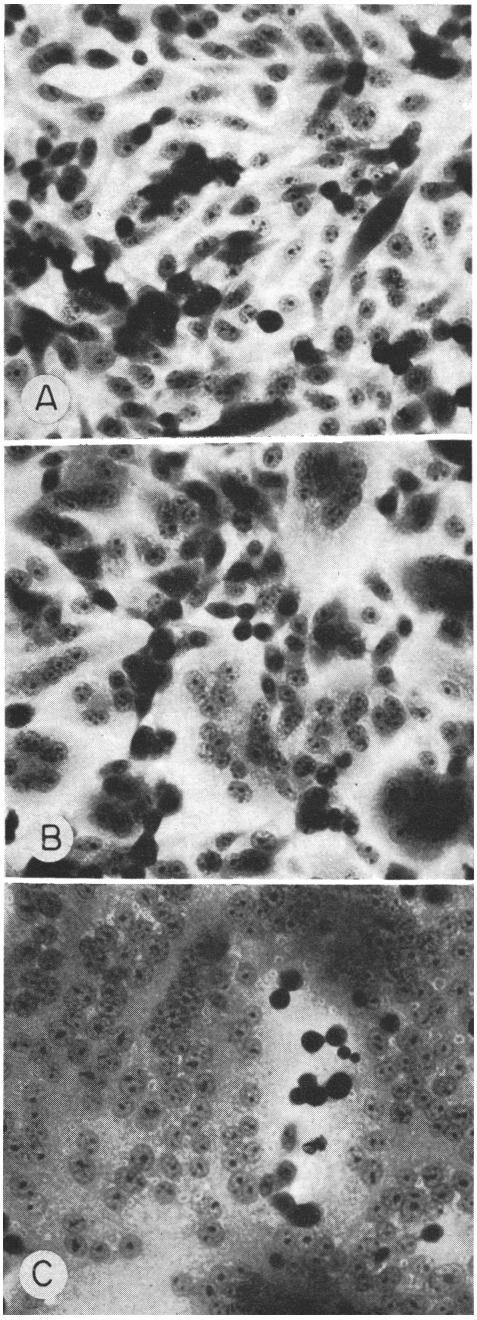

FIG. 1. Photomicrographs of monolayers of BHK21-F cells showing fusion induced by SV5: hematoxylin and eosin staining; × 275. (A) Uninfected monolayer; cells are elongated and fibroblastic; (B) monolayer 90 min after inoculation with SV5 at a multiplicity of ~1000 pfu/cell; such large syncytia form rapidly in the absence of virus replication when cells are exposed to concentrated SV5; (C) monolayer 14 hrs after infection with SV5 at a multiplicity of 16 pfu/cell; virtually all cells have fused together to form a single huge syncytium.

tion of separated phospholipid species, and determination of radioactivity in the isolated fractions were carried out as described previously in detail (12–14). Protein content of homogenates was determined by the method of Lowry *et al.* (16). The water-soluble radioactivity accumulating during incubation with lysolecithin-^{32}P was identified as glycerylphosphorylcholine by established methods (17–18).

Results. Breakdown of lecithin. Experiments were carried out to determine the extent of breakdown of radioisotopically labeled lecithin by each of the following: (i) concentrated and purified SV5 virions, (ii) homogenates of uninfected control BHK21-F cells and cells infected with SV5 which fuse into a single huge syncytium by 14–18 hr after inoculation (see Fig. 1), (iii) homogenates of control BHK21-F cells and cells inoculated with sufficient concentrated SV5 to produce extensive fusion within 1 hr (see Fig. 1), and (iv) intact BHK21-F cells which had been preincubated with choline methyl-^{14}C for 22 hr and then inoculated with an amount of concentrated SV5 sufficient to produce extensive cell fusion within 1 hr. The use of either ^{32}P or choline methyl-^{14}C as labels for lecithin and thin layer chromatographic separation of the reaction products permit detection of any of the currently recognized phospholipase activities.

Figure 2 shows the results of such experiments. Concentrated virus (Fig. 2A) had virtually no hydrolytic activity toward lecithin-^{32}P either in the presence, or absence, of deoxycholate. Therefore, the virus particles themselves do not appear to possess phospholipase activity.

Incubation of homogenates of uninoculated BHK21-F cells with lecithin-^{32}P (Fig. 2B, solid circles) resulted in slight, but detectable loss of radioactivity from lecithin, which was recovered in both lysolecithin and glycerylphosphorylcholine. This breakdown of lecithin-^{32}P was *not* enhanced by exposure of

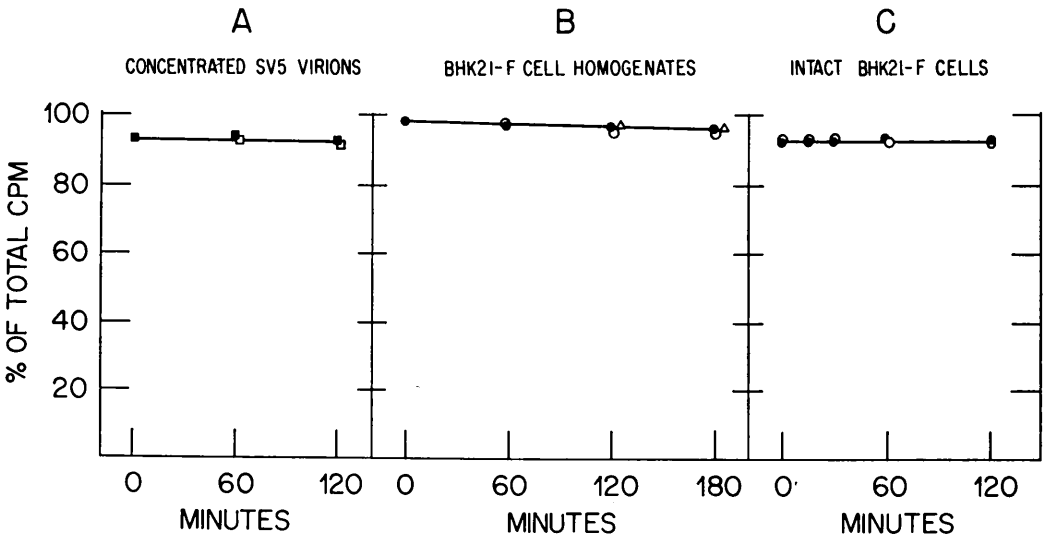

FIG. 2. Breakdown of lecithin by purified SV5 virions or by BHK21-F cells: (A) Purified SV5 virions; reaction mixtures contained 6400 hemagglutinating units of purified virus, 0.05 μmoles of lecithin-^{32}P, 7 μmoles of $CaCl_2$, and 5 μmoles of phosphate buffer, pH 7.4, in a total of 0.5 ml; (□), without sodium deoxycholate; (■), 1 mg of sodium deoxycholate added. (B) Homogenates of BHK21-F cells; reaction mixtures were prepared as described for (A) except that 0.08 μmoles of lecithin-^{32}P were used, and cell homogenates were prepared in 0.25 *M* sucrose (0.9 mg of protein/aliquot) were added. (●), Control cells incubated at 37° without virus before homogenization; (○), cells inoculated with concentrated SV5, ~1000 pfu/cell, and incubated for 90 min at 37° before homogenization; (△), cells infected with SV5 (16 pfu/cell) and incubated at 37° for 14 hr before homogenization. (C) Intact cells with prelabeled lecithin; cells were incubated for 22 hr with 0.2 μCi of choline methyl-^{14}C/monolayer. The radioactive medium was removed, the cells were washed twice and incubated for 2 hr with unlabeled medium, and then further incubated with (○) and without (●) concentrated SV5, ~1000 pfu/cell.

cells to concentrated virus (~1000 pfu/cell) for 1.5 hr prior to homogenization (open circles) or by infecting cells with an inoculum of 16 pfu/cell and harvesting 14 hr later (triangles), although both of these procedures caused extensive cell fusion (see Fig. 1).

The above experiments suggest that virus-induced cell fusion is not accompanied by an accelerated breakdown of lecithin. However, one could argue that the lecithin-^{32}P added to the assay mixture did not adequately represent the lecithin of the cell membrane. To investigate this possibility endogenous lecithin of BHK21-F cells was labeled by incubation with choline methyl-^{14}C. The labeled cells were then transferred to nonradioactive medium and reincubated with (Fig. 2C), and without, concentrated SV5. In spite of the membrane changes associated with extensive cell fusion, essentially no breakdown of labeled cellular lecithin took place. Total radioactivity in phospholipid, radioactivity in sphingomyelin (~6% of the total), and that in lysolecithin (2% of the total) also remained unchanged throughout the incubation period.

Lysolecithin metabolism. Degradation of phospholipid might affect membrane integrity, and therefore possibly play a role in cell fusion, in two ways: (i) by removing important membrane constituents, and (ii) by formation and accumulation of breakdown products, such as lysolecithin, that possess potent membrane-lytic properties. In the mammalian tissues and cell types thus far investigated, degradation of lecithin takes place through the action of phospholipase A, resulting in formation of lysolecithin, which may then be further de-acylated by lysolecithinase to water soluble, and nonlytic,

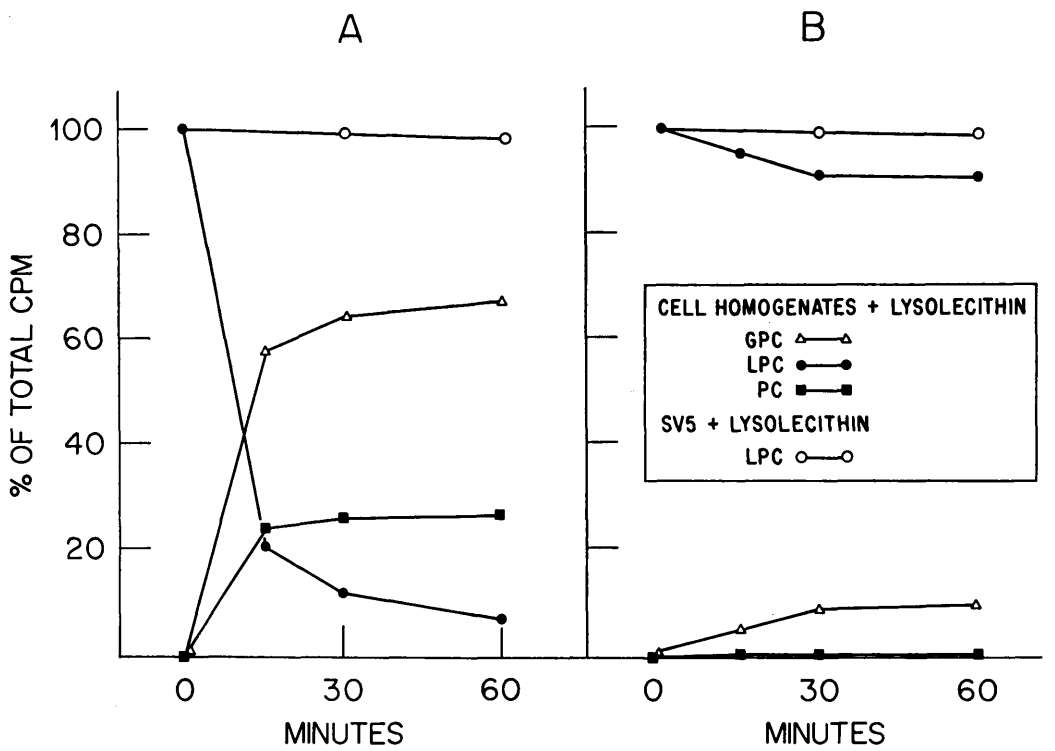

FIG. 3. Metabolism of lysolecithin at low (A) and high (B) concentrations by purified SV5 virions or by uninfected BHK21-F cells; reaction mixtures contained either 0.045 μmoles (A) or 4.4 μmoles (B) of lysolecithin-^{32}P, 5 μmoles of phosphate buffer, pH 7.4, and either 6400 hemagglutinating units of SV5 or BHK21-F cell homogenate, (2.5 mg of protein/aliquot). To the tube that contained the low concentration of lysolecithin, 5 μmoles of ATP, 5 μmoles of $MgCl_2$, and 0.1 μmoles of CoA were added.

glycerylphosphorylcholine (12–14, 19–22). The studies of Lands and co-workers (23, 24), and of Erbland and Marinetti (25–27) have revealed that lysolecithin can also be reconverted to lecithin by two pathways: (i) by direct acylation of lysolecithin, a reaction which requires ATP, CoA, and free fatty acid, and (ii) by transfer of a fatty acid from one lysolecithin molecule to another, a reaction which requires high lysolecithin concentration but no ATP and CoA (12, 13, 27). These pathways of lecithin synthesis have been demonstrated to coexist in homogenates of some cell types, but not of others (13, 24, 26).

The experiments described above showed that extensive breakdown of lecithin did not occur during cell fusion. However, low concentrations of lysolecithin are strongly lytic (28). It was therefore of interest to determine whether BHK21-F cells possess enzymes which would be capable of removing lysolecithin if accumulation, perhaps in discrete areas of the cell, did take place. The direct effect of concentrated virus on lysolecithin was also investigated.

Figure 3 shows the metabolism of lysolecithin-^{32}P by concentrated SV5 and by homogenates of uninfected BHK21-F cells at low and high lysolecithin concentrations. At low lysolecithin concentration, (Fig. 3A), addition of concentrated virus (open circles) resulted in minimal degradation of lysolecithin and no formation of lecithin. Homogenates of BHK21-F cells manifested pronounced lysolecithinase activity, indicated by the fall in lysolecithin (closed circles) and the appearance of glycerylphosphorylcholine (triangles). In addition, lysolecithin was converted to lecithin (solid squares). Lecithin syn-

thesis leveled off, however, after 15 min presumably in part because of the rapid disappearance of substrate.

At high lysolecithin concentration (Fig. 3B), there was no detectable metabolism of lysolecithin by the concentrated virus. Homogenates of BHK21-F cells showed considerable lysolecithinase, but virtually no lecithin forming activity. These experiments at high substrate concentration were carried out to assess the second pathway of lecithin formation from lysolecithin, i.e., the transfer reaction, and therefore the cofactors (ATP and CoA) needed for direct acylation of lysolecithin, were omitted from the reaction mixture. It thus appears that BHK21-F cells possess the enzyme necessary for the acylation reaction, but may lack the ability to transfer fatty acids from one molecule of lysolecithin to another.

The effect of exogenous lysolecithin on BHK21-F cells was also investigated. Serial twofold dilutions of lysolecithin in reinforced Eagle's medium with 2% calf serum were added to cell monolayers and morphological changes were followed by phase contrast microscopy. At lysolecithin concentrations of 1.4 m*M* or higher the cells rounded up within 5–10 min and lysed soon thereafter; at concentrations of 0.7 m*M* or lower no changes were detected. No cell fusion was observed at any concentration of lysolecithin. The lack of morphological evidence of cell damage at concentrations up to 0.7 m*M* may in part be explained by the above demonstrated ability of the BHK21-F cell to remove lysolecithin by a highly active lysolecithinase or by conversion to lecithin.

Discussion. These results indicate that concentrated, purified SV5 virions apparently do not possess phospholipase activities. On the other hand, homogenates of BHK21-F cells do exhibit lecithin- and lysolecithin-splitting activities. Hydrolysis of lecithin under our experimental conditions is slow, and neither affected by short exposure of the cells to concentrated virus, nor by multiplication of SV5 during a 14–18-hr period, both conditions that produce widespread alterations of cell membranes which result in cell fusion. Of particular significance is the observation that the lecithin of intact cells, labeled during preincubation with choline methyl-^{14}C, does not undergo appreciable hydrolysis whether or not virus-induced changes take place. Further, the finding of an active lysolecithinase, and an enzyme capable of directly converting lysolecithin to lecithin, indicates that BHK21-F cells do possess pathways for elimination of this strongly membrane-lytic agent. Finally, the failure of exogenous lysolecithin to cause cell fusion provides additional suggestive evidence that this compound is not an important factor in virus-induced cell fusion.

Falke *et al.* (29) have previously considered the possibility that accumulation of lysocompounds might play a role in fusion of rabbit kidney cells induced by herpes simplex virus infection. These authors fractionated cellular phospholipids labeled during preincubation with oleic acid-1-^{14}C and found no accumulation of lysolecithin during cell fusion. Thus, although different viruses were used which may differ somewhat in their cell fusing activities (3), our results with SV5 are in agreement with the previous work with herpes simplex virus with regard to lack of evidence for a role of lysolecithin in virus-induced cell fusion.

Summary. Homogenates of BHK21-F cells which fused rapidly after inoculation with a high concentration of the parainfluenza virus SV5, or which fused more slowly with SV5 infection and virus multiplication, showed no greater breakdown of lecithin than uninoculated control cells. Cellular lecithin did not undergo appreciable hydrolysis in intact cells under conditions in which extensive cell fusion occurred. BHK21-F cells possess lysolecithinase activity, and the ability to convert lysolecithin to lecithin. The cells thus possess the pathways for elimination of the membrane-lytic agent lysolecithin. No phospholipase activities were found associated with purified SV5 virions. We conclude that the cell fusion induced by this virus is not due to an effect on a major structural lipid such as lecithin.

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