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Interaction of Polyunsaturated Fatty Acids with Animal Cells and Enveloped Viruses

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Essential unsaturated fatty acids such as oleic, linoleic, or arachidonic were incorporated into the phospholipids of animal cells and induced in them a change in the fluidity of their membranes. Exposure of enveloped viruses such as arbo-, myxo-, paramyxo-, or herpesviruses to micromolar concentrations of these fatty acids (which are not toxic to animal cells) caused rapid loss of infectivity of these viruses. Naked viruses such as encephalomyocarditis virus, polio virus or simian virus 40 were not affected by incubation with linoleic acid. The loss of infectivity was attributed to a disruption of the lipoprotein envelope of these virions, as observed in an electron microscope.

Enveloped viruses, which mature by budding through the plasma membrane of their host cells, have a lipid pattern similar to that of the membrane of the host cell (7). Thus, it is possible to obtain viruses with the same proteins but different lipid patterns by growing a given virus in different cell types. There is, however, also a possibility that the lipid composition of some enveloped viruses is determined to a certain extent by the proteins coded by the virus and incorporated into the cell membrane before or during the "budding" process (26). Membrane rigidity is then determined by the protein-lipid interactions. In fact, the membrane "rigidity" of the viral envelope, which is determined by its lipid composition, is significantly higher than that of the host cell plasma membrane (5, 6, 10, 12, 16, 18). These observations indicate that at the time of formation of the viral envelope the spatial organization of host cell lipids around the virus-specific proteins is selective (22). The biological significance of the increased rigidity of the viral envelope, due to an increase in the relative amount of its rigid lipids, is still obscure. We have used unsaturated fatty acids to fluidize host cell plasma membranes to study the production of animal viruses in such modified cells.

In this study, we demonstrate that polyunsaturated fatty acids, which decrease rigidity of biological membranes at concentrations harmless to animal cells, destroy enveloped viruses by "dissolving" their envelopes.

MATERIALS AND METHODS

Viruses, cells, and media. The following viruses were used; Myxoviruses (influenza A, Mel.), paramyxoviruses (Sendai and Newcastle disease viruses), arboviruses (Sindbis and West Nile), picorna viruses (poliovirus strain mEF2 and encephalomyocarditis [EMC]), papova simian virus 40 (SV40), and herpes virus types 1 and 2.

Myxo- and paramyxoviruses were grown in 10-day-old embryonated eggs, arboviruses were grown in BHK-21 cells (Eagle medium F-12, GIBCO Laboratories, Grand Island, N.Y.), EMC virus was grown in mouse brains, poliovirus was grown in the monkey line BGM (M-199 medium), herpesviruses were grown in Vero cells (M-199 medium), and SV40 was grown in the monkey B-SC$_1$ cell line.

Fatty acids. Oleic acid (cis-9-octadecenoic acid), linoleic acid (cis-9-cis-12-octadecadienoic acid), and arachidonic acid (5, 8, 11, 14-eicosatetraenoic acid from procine liver, Sigma Chemical Co.) were used at concentrations of 5 to 100 μg/ml. Stock solutions of 1 mg/ml were prepared in ethanol, and from this solution dilutions were made in phosphate-buffered saline (PBS), pH 7.2. For studying the incorporation of linoleic acid into animal cells [1-^{14}C]linoleic acid (51 mCi/mmol, Amersham) was used.

Electron microscopy. Sindbis and Sendai virus suspensions treated with linoleic acid (10 μg/ml) for 5 to 10 min were deposited on electron microscope grids, stained with sodium phosphotungstate (pH 7.4), and examined in a JEM-100B transmission electron microscope.

Fluidity measurements. Suspensions of control as well as of cells treated with unsaturated fatty acids were labeled with 1,6-diphenyl-1,3,5-hexatriene (DPH) (23). Two milliliters of freshly prepared suspension of DPH (0.1 ml of DPH in tetrahydrofuran [2×10^{-3}M] in 100 ml of PBS) was added to a pellet of trypsinized and washed cells at a final concentration of 10^6 cells per ml and incubated at room temperature for 20 min. The cells were then washed twice in PBS, and their fluorescence polarization was determined with the aid of an MV-1 Elscint Microviscosimeter (8), which takes readings at two independent cross-polarized channels and directly shows the polarization values (P).

Experimental procedure. Two types of procedures were employed. In one, control BHK cells as well as cells treated with unsaturated fatty acids were

infected with Sindbis virus at a multiplicity of infection of 1 to 10 plaque-forming units (PFU) per cell at different time intervals before or after the treatment with fatty acids, and the yield of the virus in cultures was assayed by plaque counts after 10 h of incubation. In the other procedure, various viruses representing different classes were incubated with unsaturated fatty acids at different time and concentration schedules, and the infectivity of the treated viruses was determined in an appropriate assay system.

RESULTS

It was shown that linoleic acid, at a concentration which did not affect cell growth (2), inactivated enveloped viruses, but was innocuous to "naked" viruses (20; A. Kohn and M. Inbar, Israeli patent no. 58109, 24 August 1979). In our experiments linoleic acid in concentrations up to 25 $\mu g/10^6$ cells per ml did not affect the growth of BHK cells (Fig. 1). The cells in monolayer multiplied to the same extent as control cells and incorporated [^{3}H]thymidine equally well. Similarly arachidonic and oleic acids did not interfere with the growth of BHK cells when tested over the same range of concentrations (data not shown). Linoleic acid, being an essential fatty acid, is taken up by the BHK cell and metabolized into phospholipids. After a 6-h incubation period with linoleic acid, the cells incorporated 97% of the acid into phospholipids and glycerides, the major fraction (50 to 55%) appearing as phosphatidyl choline (data not shown). The results correspond well with data of Williams et al. (27), who used Tween fatty acids esters as a supplement in growth medium. The remaining 3% of free fatty acids may exist as micellar or vesicular forms in aqueous medium. The critical micelle concentration is in the range 10^{-6} to 10^{-3} M; thus, in the particular case of linoleic acid, it can be calculated that only 1% would be in the form of monomers (18, 24).

The uptake of [^{14}C]linoleic acid (Fig. 2) was accompanied by a drop in the degree of fluorescence polarization of the cellular lipids. This change was not affected by adsorption of Sindbis virus to the cells treated beforehand with linoleic acid (Fig. 3), i.e., although the virus per se affected slightly the "fluidity" of control cells, it had no effect on the fluorescence polarization of cells incubated with linoleic acid for 1 to 6 h.

HeLa cells which were treated with linoleic acid with resultant decreased microviscosity (decrease in the P values) of their membranes adsorbed Sendai virus and fused to the same extent as control, untreated cells (Table 1). This experiment indicates that the change in the membrane viscosity does not affect the affinity or specificity of the neuraminic receptors for the viruses on the cell surface.

The yield of Sindbis virus-infected cells treated with linoleic acid depended on the time of the infection in relation to addition of the fatty acid. It was however, independent of the actual microviscosity of the host cell plasma membrane at the time of infection. BHK cells were treated with 10 μg (in 1 ml) of linoleic acid for 0 to 3 h before Sindbis virus infection, or 30 min to 3 h after virus infection. A 0.2-ml amount of 2×10^7 PFU of Sindbis virus was added to

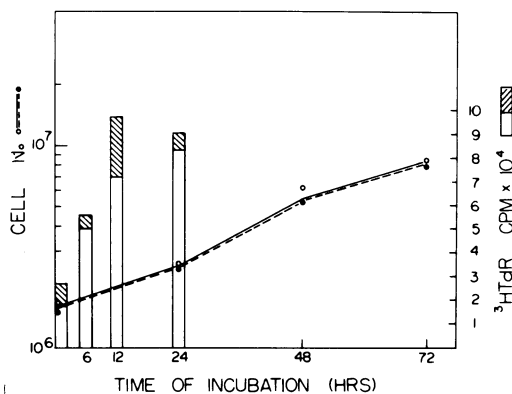

FIG. 1. *Effect of linoleic acid on the growth of BHK cells. BHK cells were incubated with 3 μCi of [^{3}H]thymidine in the presence or absence of linoleic acid (10 μg/ml) in parallel dishes. At times indicated, cultures were washed, cells were removed by trypsin, and the suspension was counted in a hemocytometer and then dissolved in sodium dodecylsulfate and counted in a scintillation counter. Open symbols, with linoleic acid; shaded symbols, without linoleic acid.*

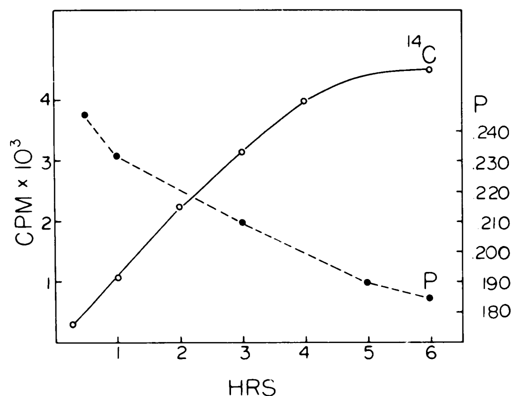

FIG. 2. *Uptake of [^{14}C]linoleic acid and its effects of microviscosity of BHK cells. 10^6 BHK cells grown for 24 h in 60-mm dishes, were treated with 10 μg (1.8 μCi) of [^{14}C]linoleic acid. After intervals, the radioactivity in the cell monolayers after trypsinization and dissolution in sodium dodecyl sulfate was determined. Suspensions of labeled cells were incubated with DPH, and their microviscosity was measured in a microviscosimeter.*

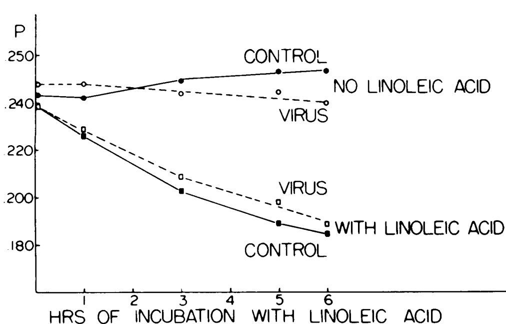

FIG. 3. *Effects of Sindbis virus adsorption on changes in microviscosity induced by linoleic acid in BHK cells. BHK cells were treated for 1 h with 1 ml of linoleic acid (10 μg/ml). The acid was then removed, and 2 × 10^7 PFU of Sindbis virus (in 0.2 ml) were added to each culture. After 45 min the cell monolayers were washed in PBS medium containing or lacking linoleic acid (1 ml) and incubated for the times indicated. Cells were removed by trypsinization, the suspension in PBS was labeled for 20 min with DPH, and the fluorescence polarization was measured in a microviscosimeter at 25°C.*

TABLE 1. *Effect of linoleic acid on cell fusion induced by Sendai virus*

Cell treatment			
Linoleic acid (10 μg/ml)	Sendai virus (800 HA/0.2 ml)	*P*	Fusion
−	+	0.264	+++
	−	0.261	−
+	+	0.220	+++
	−	0.222	−

[a] HeLa cells monolayers in 60-mm polystyrene dishes were incubated with 4 ml of linoleic acid (10 μg/ml) for 5 h. The medium was then drained, and Sendai virus (800 hemagglutinating units (HA) in 0.2 ml) was added to control in linoleic acid-treated cells. After 30 min at 37°C, the 4 ml of medium was replaced on the cell monolayers and the incubation at 37°C continued for an additional 2.5 h. The cells were then washed in PBS, fixed in absolute methanol, and stained with Giemsa. Fusion was determined by microscopical examination. +++ denotes almost confluent fusion of cells. From parallel control and treated cultures, measurements of fluorescence polarization (P) were made.

washed monolayers for 35 min, whereupon medium with or without linoleic acid was replaced in the dish (in 1 ml). The yield of Sindbis virus in the culture was assayed in BHK monolayers after 10 h of incubation of the test cultures. (Microviscosity of the cells treated with linoleic acid (trypsinized and DPH labeled) dropped from $P = 0.260$ to $P = 0.172$ to 0.190 ($\bar{x} = 0.187$, standard deviation [SD] = 0.076). The virus yield in cells treated with linoleic acid 1 to 3 h before the infection was the same as in untreated controls (2.4 × 10^9 PFU/ml, SD = 6.3 × 10^8). Simultaneous addition of linoleic acid and of the virus to the cells or additions within 30 min of each other resulted in a decrease of the virus yield to about 7.5 × 10^8 PFU/ml (SD = 5 × 10^8). Since the determination of infectivity was performed 10 h after the infection, i.e., the end of the first cycle of replication, the amount of virus produced was proportional to the infective virus input. These findings suggested that it was the direct interaction between the free fatty acid and the virus that caused the loss of viral infectivity. This deduction was tested in an experiment in which the enveloped viruses were directly incubated in vitro with linoleic acid for different periods of time, and their infectivity was then assayed in the appropriate cell system.

The results (Table 2) indicate that an incubation period of 15 to 30 min for deoxyribonucleic acid and ribonucleic acid enveloped viruses with 10 to 20 μg of linoleic acid resulted in a marked reduction in their infectivity. However, naked viruses, such as poliovirus, EMC virus, or SV40 were not affected by a similar treatment.

Inspection of enveloped viruses treated with linoleic acid after negative staining in an electron microscope indicated that within a few minutes of adding the linoleic acid to the viruses (Sendai or Sindbis) their envelopes were damaged and destroyed (Fig. 4). Nevertheless, the hemagglutinating viruses such as influenza, Newcastle disease, Sendai, or Sindbis viruses that lost their infectivity because of the disruption of their envelopes still had functional hemagglutinins. Paramyxoviruses, however, lost their fusing properties, as expected, since the integrity of the lipid phase of the envelope was shown to be essential for fusion (3).

Other unsaturated fatty acids, such as arachidonic and oleic acids (but not saturated acids, such as stearic acid) also inactivated Sindbis virus proportionally to their concentration (Fig. 5). Other viruses as well, such as influenza and Sendai (titrated in embryonated eggs), lost their infectivity when incubated for 15 to 18 min with 10 μg of any of the unsaturated fatty acids, but were not affected by the saturated stearic acid (Table 3).

It was of interest to elucidate why the viral envelopes were destroyed by unsaturated fatty acids at concentrations which were innocuous to animal cells. One explanation might be that the metabolically active cells take up the fatty acids and metabolize them to structural phospholipids, whereas metabolically inactive viruses cannot deal with these acids: the fluidization of the viral envelope causes its disruption.

If this assumption is correct, one would also expect the disruption of animal cells in which

TABLE 2. *Effects of linoleic acid on the infectivity of viruses*[a]

Virus type	Nucleic acid	Virus	Time of incubation (min) at 25°C	Test cells	Virus infectivity[b]	
					Control (log)	Decrease (log)
Enveloped	RNA	Sendai	10	Eggs	8.3	2.8
			15		7.5	1.8
			60		8.3	4.8
		NDV	15	Eggs	8.0	2.5
		Influenza A_1 (MEL)[c]	30	Eggs	6.0	>3.0
		Influenza A_1 (PC E2)	15		5.7	>4.2
		Sindbis	5	BHK	8.0	3.0
			10		7.5	3.5
					7.3	3.8
			45			>5.5
		West Nile	30	BHK	7.1	3.8
	DNA	Herpesvirus type 1 (VR)	30	Vero	6.5	4.2
		Herpesvirus type 2 (Rapp)	30		6.5	>4.2
Naked	RNA	Poliovirus 1[c]	45	BGM	8.5	0
		EMC	45	L 929	8.6	0
	DNA	SV40	30	BSC	8.0	0

[a] Virus suspension in 1 ml of PBS was mixed with 10 μg of linoleic acid and incubated at room temperature for times indicated. Serial dilutions of the suspension were made and titrated in appropriate cell systems. RNA, Ribonucleic acid; DNA, deoxyribonucleic acid.

[b] Virus infectivity is expressed as 50% egg-infective doses and as PFU in cell monolayers.

[c] Concentration of linoleic acid in this experiment was 20 μg/ml.

the synthetic processes are halted, such as in the case of erythrocytes. We compared therefore the results of treatment of erythrocytes of various species with linoleic acid and their ability to retain hemoglobin, as a measure for the state of their membranes. We found that at 4 and 20°C linoleic acid (at 10 μg/ml) would not hemolyse erythrocytes of human, sheep, or chicken origin, but at 37°C the results for the various species differed. Whereas the sheep and human erythrocytes were lysed almost completely, the effect on chicken erythrocytes was very small (Fig. 6). The sheep and human erythrocyte membranes thus became leaky to hemoglobin at 37°C, a result of their fluidization by the unsaturated fatty acid. In this respect the membrane of erythrocytes seem to resemble more the viral envelope than the membrane of metabolically active animal cells, which maintain the ionic barrier in spite of its fluidization by unsaturated fatty acid.

DISCUSSION

The experiments of Sands et al. (21) and Reinhardt et al. (17) on the effects of unsaturated fatty acids on phage PR4 led them to conclude that these compounds blocked the entry of the phage deoxyribonucleic acid into the cells. Later Sands et al. (20) demonstrated that treatment of PM2 phage with monopalmitolein resulted in complete disassembly of the phage (sucrose gradient velocity sedimentation of ^{32}P labeled phage). Sands and co-workers also showed that unsaturated monoglycerides and alcohols inactivated enveloped animal viruses such as herpesviruses (19).

In our experiments we have demonstrated a direct effect of unsaturated fatty acids on the lipid envelope of a variety of animal viruses. Their envelopes disintegrated under the influence of unsaturated fatty acids such as linoleic, oleic, or arachidonic, but were not affected by stearic acid. The loss of the envelope in these viruses accounts for the loss of their infectivity.

We have also observed that linoleic acid caused damage to mammalian erythrocytes when incubated at 37°C (but not at 4°C). At the concentration used (5 to 25 μg/ml), the unsaturated fatty acids were found to be innocuous to animal cells in culture, but were harmful to some erythrocytes and to enveloped viruses. This finding can be explained by the ability of nucleated animal cells to process the unsaturated fatty acids and to incorporate them into their phospholipids. On the other hand, viruses and erythrocytes, which do not process free fatty acid, are damaged. The presence of free fatty acid in the membrane thus seems to be incompatible with its integrity. The molecular details of the interaction between the free fatty acids and the viral envelope in comparison with their behavior in cell membrane bilayers are still obscure.

FIG. 4. *Electromicrographs of viruses treated with linoleic acid. Sendai virus before (A) and after (A-L) treatment with 10 μg of linoleic acid per ml for 5 to 8 min. Sindbis virus before (B) and after (B-L) identical treatment. The virus samples were stained with 2% phosphotungstate and examined in JEM-100B electron microscope. Magnification: ×100,000.*

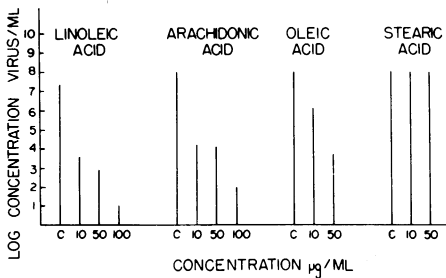

FIG. 5. *Effects of unsaturated and saturated free fatty acids on Sindbis virus. To a suspension of Sindbis virus (2 × 10^7 PFU/ml) the following free fatty acids were added at the concentrations indicated on the abscissa: linoleic, arachidonic, oleic, and stearic. After 15 min of incubation, the virus was titrated on BHK cells.*

However, the fact that naked viruses lacking lipid compounds are not affected by the unsaturated free fatty acids indicates that it is the lipid envelope which is the target for the fatty acids.

The lipid composition of viral membranes, although remarkably similar to that of the membrane of their host cells, is nevertheless quantitatively different. The differences are in the ratio of sphingomyelin to phosphatidylcholine, the ratio of saturated to unsaturated fatty acids, and the ratio of cholesterol to phospholipids, all of which are significantly higher in the virus envelope than that in the cell plasma membrane. These differences account for the higher rigidity of the viral envelope in relation to that of the plasma membrane (1, 4, 14, 15, 24). It is not clear yet whether this increased rigidity of the viral envelopes is required for an effective viral infec-

TABLE 3. *Effects of unsaturated and saturated fatty acids on enveloped viruses*[a]

Virus	Log reduction (15 min)				Control log virus concn
	Linoleic acid	Arachidonic acid	Oleic acid	Stearic acid	
Influenza A_1 (PC-E2)	4.2	ND	4.0	0.5	5.7
Sindbis	4.0	3.8	6.1	0	8.0
Sendai	1.8	1.8	ND	ND	7.5

[a] The viruses were incubated with 10 μg of fatty acid per ml for 15 to 18 min, whereupon their infectivity was determined in appropriate cell system (see Table 2).

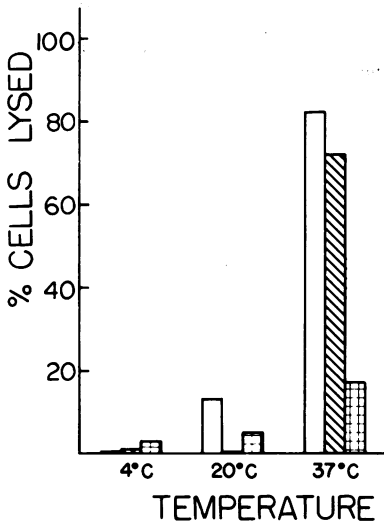

FIG. 6. *Effects of linoleic acid (10 μg/ml) on erythrocytes from various species of animals. Linoleic acid (10 μg/ml) was added to a 1% suspension of human, sheep, or chicken erythrocytes and incubated for 10 min at the indicated temperatures. At the end of the incubation period, the erythrocytes were centrifuged and the hemolysis in the supernatant was measured in a spectrophotometer at 450 nm. □ Human; ▧ sheep; ▦ chick.*

tion and virulence. The available data (9, 12) seem to indicate that this might be the case. Cholesterol depletion of vesicular stomatitis virus, accompanied by a decrease in the rigidity of the envelope of this virus, resulted in a substantial loss of vesicular stomatis virus infectivity (12).

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