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The Effect of Octadecenoic Acid on the Growth of Japanese Encephalitis Virus in Novikoff Hepatoma Cells (39094)

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Previous experiments in this laboratory (1) have shown that the growth characteristics of Novikoff hepatoma cells are dependent on the type of fatty acid which is present in the growth medium. Preliminary experiments with Novikoff hepatoma cells infected with Japanese encephalitis virus (JEV), a lipid containing virus, suggested that its growth characteristics are also dependent on the type of fatty acid in the growth medium. Therefore, the following study was undertaken to investigate these effects in further detail.

Materials and methods. Cells. Novikoff rat hepatoma (strain N1S1-67) cells (2) (a gift from Dr. Peter Plagemann, University of Minnesota) were grown using a shaker culture system according to the methods described by Plagemann and Swim (2) and Steele and Jenkin (3), using Swim's 67-G medium (Grand Island Biological Co., Grand Island, N. J.).

Baby hamster kidney (BHK-21) cells (obtained from Dr. T. B. Stim, Yale Arbovirus Research Unit, Yale University, New Haven, Conn.) were cultivated according to the methods of Makino and Jenkin (4).

Virus. Japanese encephalitis virus (JEV), strain M5/596, from the 10th suckling mouse brain passage, was kindly supplied by Dr. S. Grossberg, Medical College of Wisconsin, Milwaukee, Wis. Virus seed used for the infection of Novikoff hepatoma cells was adapted by passing JEV three times in Novikoff hepatoma cells and harvesting the virus 48 hr after infection with each serial passage. Fifteen percent fetal calf serum (inactivated at 56° for 30 min) was added to the supernatant fluid, and the virus seed was stored at −70° until used. One virus pool was used throughout the experiments.

Viral infection of the hepatoma cells was performed by shaking the Novikoff hepatoma cells (1×10^6 cells/ml) and the virus (multiplicity of infection was 7) in 2 ml medium for 2 hr at 37°. At the end of the adsorption period, enough Swim's 67-G medium was added to bring the final cell number to 2×10^5 per ml. The incubation was continued at 37° in a water bath shaker.

Stability of virus in growth medium. Stability was tested in fresh and spent Swim's 67-G medium. The spent medium was prepared by inoculating the growth medium containing either 0, 25, or 125 μg 6–18:1 per ml with 2×10^5 Novikoff hepatoma cells per ml and incubating the mixture in a shaker/water bath at 37° for 48 hr. At the end of this period, the cells were sedimented at 500*g* for 10 min at 22°. The stability of viral particles in the supernatant fluid was tested in the cell-free spent medium and also in cell-free fresh medium containing either 0, 25, or 125 μg 6–18:1 per ml by further incubation at 37° in a shaker/water bath. All experiments were performed in triplicate.

Virus assay. Viral infectivity was determined in the supernatant fluid of the medium at various intervals of time after the cells had been sedimented at 500*g* for 10 min at 4°. Infectivity of the virus was determined by a plaque assay performed in triplicate from at least three independent experiments by the method of Schulze and Schlesinger (5). Twenty-four-hour-old monolayers of BHK-21 cells (4×10^5 cells/well) were used in the assay employing plastic plates with six wells (Linbro Chemical Co., Inc., New Haven, Conn.).

The methyl cellulose (4,000 cps.) used in the overlay medium was obtained from the Dow Chemical Co., Midland, Mich. The cells were stained with crystal violet, according to the method of Holland and McLaren (6), and plaque numbers were counted.

Cell enumeration. A hemocytometer was

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used to measure cell viability by performing quadruplicate counts from a minimum of three shaker flasks (7). Viability counts were performed by employing the trypan blue dye exclusion test.

Fatty acids. *Cis*-6-octadecenoic (6–18:1) and *cis*-9-octadecenoic (9–18:1) fatty acids were obtained from the Lipids Preparation Laboratory, The Hormel Institute, Austin, Minn. The sodium salts of the fatty acids were prepared and added to the medium by the methods described previously (3).

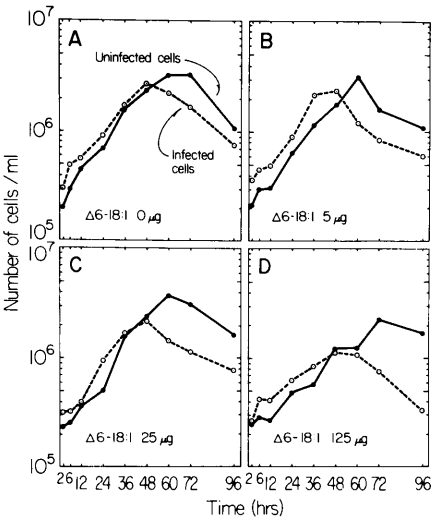

FIG. 1. Effect of Japanese encephalitis virus on the growth of Novikoff hepatoma cells in medium containing various amounts of the *cis*-6-octadecenoic acid.

Results. Infection of Novikoff hepatoma cells by JEV resulted in lower cell numbers and a shorter time to reach maximum cell numbers compared to the uninfected cells (see Fig. 1). Addition of 5 or 25 μg *cis*-6-octadecenoic acid (6–18:1) to the medium did not significantly alter the shape of the growth curve of the cells when compared to medium without 6–18:1. However, the addition of 125 μg 6–18:1 per ml of medium resulted in a sharp decrease in cell numbers in both infected and uninfected cells.

The number of infectious viral particles (PFU/ml) was enhanced when the growth medium was enriched with 5 or 25 μg 6–18:1 per ml, whereas the viral infectivity was drastically reduced during a 48-hr incubation period when the concentration of 6–18:1 in the medium was increased to 125 μg per ml. The data given in Table I show that the maximum number of infectious viral particles produced by each cell in 48 hr when the medium contained either 5 or 25 μg 6–18:1 per ml was approximately two to eightfold different than that obtained when the medium contained 0 or 125 μg 6–18:1 per ml, respectively.

The infection of cells grown in medium containing 0, 5, or 25 μg 6–18:1 resulted in a rapid increase in cell proliferation during the first 2 hr after infection (Fig. 1).

TABLE I. THE RATIO OF INFECTIVITY OF JAPANESE ENCEPHALITIS VIRUS TO VIABLE NOVIKOFF RAT HEPATOMA CELLS GROWN IN THE PRESENCE OF 6-OCTADECENOIC ACID (6–18:1)

Time (hr)	μg 6–18:1 per ml medium			
	0	5	25	125
0	2.0[a]	2.0	2.0	2.0
2				
6	<1	<1	<1	<1
12				
24	2 ± 0.4[b]	3 ± 0.8	3 ± 0.9	<1
36	14 ± 3.0	24 ± 2.1	38 ± 5.7	4 ± 3.0
48	83 ± 4.1	176 ± 5.1	193 ± 6.2	20 ± 4.1
60	43 ± 3.9	183 ± 6.3	197 ± 7.1	50 ± 3.9
72	17 ± 3.1	66 ± 3.7	110 ± 7.8	44 ± 5.0
96	2 ± 1.8	33 ± 4.1	37 ± 6.2	93 ± 7.7

[a] Multiplicity of infection.

[b] Values are means of the ratio of virus infectivity to viable cells ± SE from three independent experiments ($n = 9$).

TABLE II. STABILITY OF JAPANESE ENCEPHALITIS VIRUS IN CELL-FREE FRESH AND SPENT SWIM'S 67-G MEDIUM CONTAINING *Cis*-6-OCTADECENOIC ACID (6–18:1) AT 37 DEGREES[a]

Time (hr)	Fresh medium (6–18:1/ml)			Spent medium (6–18:1/ml)		
	0	25	125	0	25	125
0	6.63[b] ± 0.02	6.63 ± 0.02	3.03[c] ± 0.51	6.62 ± 0.02	6.64 ± 0.02	6.62 ± 0.02
1	6.60 ± 0.04	6.60 ± 0.04	2.62 ± 0.66	6.59 ± 0.05	6.60 ± 0.04	6.58 ± 0.05
2	6.60 ± 0.05	6.46 ± 0.09	2.24 ± 0.70	6.59 ± 0.08	6.60 ± 0.07	6.52 ± 0.08
4	6.36 ± 0.06	6.40 ± 0.09	0	6.51 ± 0.09	6.46 ± 0.09	6.38 ± 0.10
6	6.13 ± 0.09	6.03 ± 0.09	0	6.32 ± 0.12	6.31 ± 0.15	6.19 ± 0.20
12	5.60 ± 0.16	5.29 ± 0.17	0	5.80 ± 0.24	5.83 ± 0.19	5.62 ± 0.25
24	3.79 ± 0.50	3.10 ± 0.53	0	4.75 ± 0.50	4.85 ± 0.55	3.94 ± 0.66
48	0[d]	0	0	2.75 ± 0.39	2.64 ± 0.40	1.26 ± 0.39
72	0	0	0	1.71 ± 0.33	1.32 ± 0.38	0
96	0	0	0	0	0	0

[a] See text for details of experimental conditions.

[b] Values are the means expressed as log PFU/ml ± SE of three independent experiments assayed in triplicate ($n = 9$).

[c] All flasks received the same virus input, but when the fresh medium contained 125 μg 6–18:1 per ml, the virus titer dropped precipitously in the 5 min required to take and prepare the samples for analysis.

[d] 0 = $<10^1$ PFU/ml.

The graph presented in Fig. 2 shows the greatest virus yield was obtained in the presence of 5 and 25 μg of 6–18:1. The data also suggest that the stability of viral particles was better in spent medium containing added fatty acids than in the control medium. This observation was tested as outlined under Methods and Materials, and the results are given in Table II. The titer of the virus fell off precipitously when the fresh medium contained 125 μg 6–18:1 per ml. In the 5 min required to take the sample and prepare it for assay, there was a loss of 3 logs of infectivity. However, when the medium containing 125 μg 6–18:1 per ml was first incubated with cells for 48 hr and cells were removed before the virus was added, it was found that the stability of the virus was much higher than in the fresh medium containing 125 μg 6–18:1 per ml.

When the 6–18:1 was replaced with the 9–18:1, the numbers of hepatoma cells were consistently slightly higher irrespective of whether the cells had been grown in presence or absence of either fatty acid before infection. The results of these comparisons are given in Table III.

From the data presented in Table IV, it can be seen that the number of infectious viral particles was greater when the growth

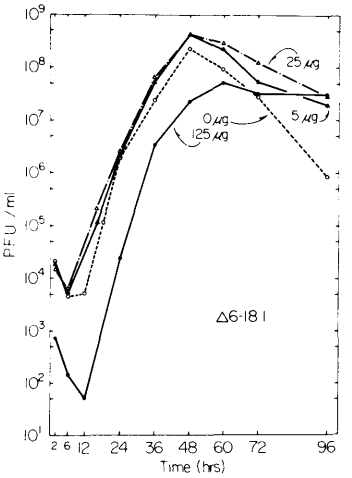

FIG. 2. The infectivity of Japanese encephalitis virus obtained (Plaque Forming Units per ml medium) from infected Novikoff hepatoma cells cultivated in Swim's 67-G medium in the presence of various amounts of *cis*-6-octadecenoic acid.

medium of the cells contained 125 μg 9–18:1 per ml than in the presence of 125 μg 6–18:1 per ml added after infection. Thus, with respect to the contro medium, the number of infectious viral particles was increased when the 9–18:1 was added to the medium and decreased when 6–18:1 was added. The drop in infectivity in the 2 and 6 hr samples

TABLE III. THE EFFECT OF INFECTION BY JAPANESE ENCEPHALITIS VIRUS ON THE VIABILITY COUNT OF NOVIKOFF HEPATOMA CELLS[a]

Time after infection (hr)	Fatty acid added to medium						
	0:0	0:6	0:9	6:0	9:0	6:6	9:9
2	5.37[c]±0.05	5.30±0.05	5.27±0.05	5.28±0.05	5.30±0.04	5.31±0.05	5.31±0.04
48	6.09 ±0.05	5.68±0.05	5.83±0.05	5.81±0.05	5.95±0.05	5.55±0.05	5.85±0.04
60	5.85 ±0.04	5.46±0.07	5.66±0.05	5.72±0.05	5.91±0.05	5.35±0.06	5.90±0.04
72	5.62 ±0.05	5.43±0.06	5.79±0.05	5.77±0.05	5.84±0.05	5.26±0.06	5.90±0.05
96	5.54 ±0.08	5.43±0.08	5.73±0.10	5.73±0.08	5.64±0.08	5.18±0.09	5.77±0.07

[a] Grown in Swim's 67-G medium supplemented with 125 μg of either *cis*-6- (6) or *cis*-9-octadecenoic (9) fatty acid.

[b] Letter before the colon indicates the addition of the fatty acid to the medium 48 hr before infection and the letter after the colon indicates addition 2 hr after the beginning of infection. The 0 notation means no fatty acid added.

[c] Values are the means of the cell viability expressed as log cells/ml ± SE from triplicate samples of three independent experiments (n = 9). All flasks received the same input of cells and virus at the beginning of infection.

TABLE IV. PLAQUE FORMING UNITS OF JAPANESE ENCEPHALITIS VIRUS PER MILLILITER MEDIUM AFTER INFECTION OF NOVIKOFF HEPATOMA CELLS[a]

Time after infection (hr)	Fatty acid added to medium						
	0:0[b]	0:6	0:9	6:0	9:0	6:6	9:9
2	5.22[c]±0.08	2.58±0.10	4.50±0.07	5.28±0.07	5.32±0.07	2.26±0.09	4.20±0.07
48	8.57 ±0.07	7.31±0.08	8.76±0.07	8.72±0.07	8.64±0.07	5.28±0.09	8.88±0.07
60	8.37 ±0.06	7.11±0.08	8.82±0.07	8.46±0.07	8.50±0.06	5.28±0.07	8.81±0.08
72	7.88 ±0.06	6.39±0.07	8.57±0.07	8.09±0.08	7.81±0.07	5.97±0.07	8.55±0.08
96	7.16 ±0.06	5.54±0.07	7.71±0.07	7.50±0.08	7.14±0.07	5.99±0.08	7.81±0.06

[a] Growing in Swim's 67-G medium supplemented with 125 μg of either *cis*-6- (6) or *cis*-9-octadecenoic acid (9) per ml.

[b] See footnotes to Table III.

[c] Values are means expressed as log PFU/ml ± SE from triplicate samples of three independent experiments (n = 9).

can be associated with loss of infectivity of JEV *in vitro* on contact with 6–18:1 in fresh medium.

The presence of 125 μg 9–18:1 in the growth medium resulted in a large increase in the number of infectious particles produced by each cell (Table V), whereas the presence of the same amount of 6–18:1 resulted in a large reduction in viral output by each cell. The continuous presence of 6–18:1 before, during, and after infection led to a very large reduction in viral multiplication.

Discussion. Henle and Chambers (8) found that chicken embryonic cells inoculated with undiluted influenza A virus had a yield of virus lower than cells inoculated with a diluted virus inoculum. Henle and Henle (9) and von Magnus (10, 11) have confirmed this effect. Comparison of the values given in Tables I and V show that this effect is also found in a different virus incubated in cells grown in culture. When the multiplicity of infection (MOI) was one, the maximum number of infectious viral particles produced by the cells grown in the control medium was approximately four times greater than when the MOI was two. This phenomenon is probably due to a large number of inactive viral particles in the less diluted inoculum which interferes with the propagation of active viruses (9, 12).

TABLE V. RATIO OF NUMBER OF PLAQUE FORMING UNITS OF JAPANESE ENCEPHALITIS VIRUS TO NUMBER OF NOVIKOFF RAT HEPATOMA CELLS[a]

Time after infection (hr)	Fatty acid added to medium						
	0:0[b]	0:6	0:9	6:0	9:0	6:6	9:9
0	1[c]	1	1	1	1	1	1
2	<1	<1	<1	<1	<1	<1	<1
48	305±5.0	42±6.3	852±9.0	813±7.1	496±6.7	<1	1077±7.7
60	332±7.0	56±7.8	1444±10.6	555±9.9	391±8.8	<1	821±9.9
72	230±6.6	9±6.1	596±7.1	174±7.8	94±9.6	5±3.0	447±9.8
96	33+6.1	2+1.0	94+7.1	59+8.2	32+5.9	6+4.0	110+9.0

[a] Growing in Swim's 67-G medium supplemented with 125 μg *cis*-6- (6) or *cis*-9-octadecenoic acid (9) per ml.

[b] See footnotes to Table III.

[c] The ratio of virus infectivity (PFU/ml) to viable counts of Novikoff hepatoma cells ± SE. The values are the results of three independent experiments performed in triplicate.

The results of the present experiment show that an addition of 5 or 25 μg fatty acid per ml medium enhanced the number of infectious viral particles produced by each cell. However, when the amount of the 6–18:1 isomer was increased to 125 μg/ml, the output of infectious viral particles was drastically reduced. In contrast, a high amount (125 μg/ml) of the 9–18:1 resulted in the largest number of viral particles being produced. One possible reason why 6–18:1 appears to be more inhibitory to viral growth at lower concentrations than the 9-isomer, may be related to the difference in their physical characteristics. For example, the melting point (mp) of the 6-isomer is much higher than that of the 9-isomer, and the solubility properties are very different. Thus, the fatty acid that has the lower mp, i.e., 9–18:1, may be more useful in meeting the requirement for membrane formation in the viral envelope. Also, the direct effect of 6–18:1 on the hepatoma cell may reduce the number of viable host cells and, therefore, reduce the number of viable JEV produced.

One possible explanation for the difference in stability of JEV in fresh and spent medium containing 125 μg 6–18:1 (see Table II) was that the cells were able to utilize large quantities of the 6–18:1 in the 48 hr prior to infection. However, they were unable to oxidize long-chain fatty acids (13). Thus, the alternative would be for the cells to utilize the 6–18:1 in the formation of complex lipid. Although it has been shown that they are able to do so (3), the cells would have to synthesize several times their own weight in complex lipids in 48 hr. This possibility seems unlikely and will be investigated in future experiments.

Summary. The growth characteristics of Japanese encephalitis virus cultivated in Novikoff hepatoma cells grown in shaker culture can be differentially altered by the presence of 6-*cis*- or *cis*-9-octadecenoic acid in Swim's 67-G medium. The addition of 125 μg of the 6-isomer per ml medium reduced the number of infectious particles produced, whereas the same amount of the 9-isomer enhanced virus production.

The virus was found to be more stable in cell-free spent medium than in fresh medium. The presence of 125 μg 6–18:1 per ml in fresh medium resulted in a rapid loss of virus infectivity.

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