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TECHNICAL MANUSCRIPT 179

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TECHNICAL MANUSCRIPT 179

PHOTOSENSITIZATION OF ARBOVIRUSES BY ACRIDINE DYES

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ABSTRACT

This paper describes the photodynamic action of proflavine and acridine orange on eastern equine encephalitis (EEE) and Venezuelan equine encephalitis (VEE) viruses. The viruses were more sensitive to the photodynamic action of acridine orange than to that of proflavine. VEE virus was slightly more resistant than EEE virus to the photodynamic action of either dye. The dye-virus complex proved to be irreversible in that the dye-treated virus remained photosensitive upon further dilution or when the excess dye was removed by dialysis or centrifugation. Supernatant fluids of actively growing cell cultures partially protected the photosensitive virus from photoinactivation. This prevented the use of photodynamic action that would differentially separate parent virus from newly synthesized progeny as has been accomplished with other viruses. The addition of reducing compounds, cysteine, glutathione, or thiourea, also protected the photosensitive virus against inactivation by light somewhat. The photoinactivation rate of infectious nucleic acid, photosensitized by these dyes, was appreciably less than that for whole virus. This suggests a second mechanism of dye action on the photoinactivation of viruses that involves the lipoprotein coat, perhaps preferentially, to the action of the dye on the nucleic acid.

I. INTRODUCTION

Various basic dyes, such as proflavine, acridine orange, and neutral red, can sensitize certain viruses so that they are inactivated when exposed to visible polychromatic light. These same dyes have no photosensitizing effect against other viruses except when they are incorporated into the viral particle during viral maturation. The sensitive group includes both DNA and RNA viruses.

The site of action of the dyes as a photosensitizer has been postulated to be the viral nucleic acid. Presumably the difference between the resistant and susceptible viral strains is simply a matter of the ability of the dye to penetrate the viral protein coat. The present paper describes the results obtained in studying the photodynamic action of proflavine and acridine orange on two groups A arboviruses, eastern equine encephalitis (EEE) and Venezuelan equine encephalitis (VEE) viruses. The results show that the dyes combine irreversibly with the viruses and further suggest that the photoinactivation may involve the lipoprotein coat.

II. MATERIALS AND METHODS

The Trinidad strain of VEE virus and the Louisiana strain of EEE virus were used in this investigation. The virus seeds were prepared from infected chick embryos homogenized as a 10% suspension in heart infusion broth. Dye-incorporated virus seeds were prepared from viral-infected chick fibroblast (CF) cultures containing 0.2 to 0.25 μg/ml of the dye in the nutrient medium, consisting of Eagle's MEM and 10% calf serum. All viral samples were assayed by the suspended plaque technique. Infectious RNA was extracted by the hot phenol procedure described by Wecker.[1] The infectious nucleic acid was assayed on CF monolayers. All viral manipulations and titrations were performed under a red light. Fluorescent daylight lamps were used in the irradiation.

III. RESULTS

The initial photoinactivation experiments were performed on viral seeds grown in the presence of proflavine in cell cultures. The results of irradiating with polychromatic light these proflavine-grown EEE and VEE viruses are shown in Table 1. Neither unlabeled virus seed was inactivated during the interval of light exposure. The proflavine-labeled virus seeds, on the other hand, were inactivated at a rapid rate.

TABLE 1. PHOTOINACTIVATION OF PROFLAVINE-GROWN EEE AND VEE VIRUSES

Virus strain	Irradiation exposure time, min				
	0	15	30	60	75
EEE	8.8[a]	ND[b]	ND[b]	ND[b]	8.9
Proflavine-labeled EEE	5.6	2.5	<1.0	-[c]	-[c]
VEE	9.5	ND[b]	ND[b]	ND[b]	9.2
Proflavine-labeled VEE	7.8	6.3	4.5	1.9	<1.0

a. pfu/ml, $\log_{10}$.
b. Not determined.
c. Too few to assay.

When the dye-labeled virus was diluted tenfold in 0.5% lactalbumin and 10% calf serum (lact CaS_{10}) medium, or Hanks' balanced salt solution (BSS) without proflavine, the rate of photoinactivation was considerably lower than in the diluent with the proflavine dye (Table 2). Further dilution did not alter the photoinactivation rate. This suggests an irreversible binding between the dye and the receptor site on the virus. This contrasts with the combination of a dye with the receptor site of bacteriophage, which is reversed upon dilution. Table 2 also shows that incubating the unlabeled EEE virus in vitro in the presence of the dye photosensitizes the virus to approximately the same extent as virus grown in the presence of dye. These data confirm the results reported by Tomita and Prince that arboviruses are photosensitized in vitro.[2]

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TABLE 3. EFFECT OF ACRIDINE ORANGE CONCENTRATION ON THE PHOTOINACTIVATION OF EEE VIRUS

Acridine orange concentration, μg/ml	Irradiation time, min		
	0	1	2
2.5	10.6[a]	5.6	2.3
0.25	10.6	5.8	2.3
0.025	10.9	9.7	8.2
0.0025	10.2	10.5	10.2

a. pfu/ml, $\log_{10}$.

Virus samples were mixed with 0.25 μg/ml acridine orange, then unbound dye was removed by dialysis overnight or by centrifuging the virus at 40,000 rpm for 1 hour, discarding the dye-containing supernatant, and resuspending the virus pellet. As shown in Table 4, the photoinactivation of this preparation was the same as when the diluent contained excess acridine orange, thus excess dye need not be present. This information confirms the previous data obtained by diluting the dye-labeled virus seed, which showed that the dye was apparently irreversibly bound to the virus receptor site.

TABLE 4. EFFECT OF REMOVING EXTRANEOUS DYE ON THE PHOTOINACTIVATION OF EEE AND VEE VIRUSES

Virus with Bound AO[a]	0.25 μg/ml AO in diluent	Irradiation time, min				
		0	1	2	5	10
EEE	No	10.0[b]	2.8	1.9	1.3	<1.0
EEE	Yes	10.0	2.6	2.3	<1.0	-
VEE	No	8.6	3.3	-	1.6	<1.0
VEE	Yes	8.2	3.6	-	1.6	<1.0

a. Acridine orange.
b. pfu/ml, $\log_{10}$.

One of the initial goals of our laboratory was to use photosensitizing dyes as tools for allowing a clear differentiation between newly synthesized virus and the original inoculum in a culture. By using a dye-labeled inoculum, we hoped to show that eclipsed or newly synthesized intracellular virus would be resistant to photoinactivation by visible light while the dye-labeled inoculum would be inactivated. The results shown in Table 5, however, demonstrate that the extracellular inoculum was only partially inactivated. Apparently something produced by the living cells provides a protective effect. This factor has not yet been identified. However, we suspect that reducing substances normally produced by the living cells are responsible, since the mechanism of photoinactivation of dye-sensitized viruses has been attributed to an oxidation reaction, which requires the presence of molecular oxygen.

TABLE 5. PHOTOINACTIVATION OF ACRIDINE ORANGE - LABELED EEE VIRUS IN THE PRESENCE OF CELL CULTURES

Diluent	Time of visible light exposure, min		
	0	1	2
Control medium (no cells)	6.7[a]	2.8	<1.0
15 minutes after inoculating cell cultures[b]	6.2	5.5	5.2
1 hour after inoculating cell cultures[b]	7.1	5.6	5.3

a. pfu/ml, $\log_{10}$.
b. Cells present during light exposure.

In the next experiment, therefore, several reducing compounds were added to the photosensitized viral suspensions to check the compounds protective abilities during exposure to visible light. Thiourea, cysteine, and glutathione were chosen and used at a concentration of 1 μg/ml. The mixtures were incubated with the sensitized virus for 30 minutes before exposure to light. The results of this experiment are shown in Figure 1. The control photosensitized EEE virus was rapidly photoinactivated beyond the sensitivity of the assay system within a two-minute period. When the photosensitized virus was incubated with cysteine, it was completely protected against photoinactivation during the two-minute interval. Glutathione protected more than 7 logs of virus from being photoinactivated and thiourea protected 3 logs of photosensitized virus.

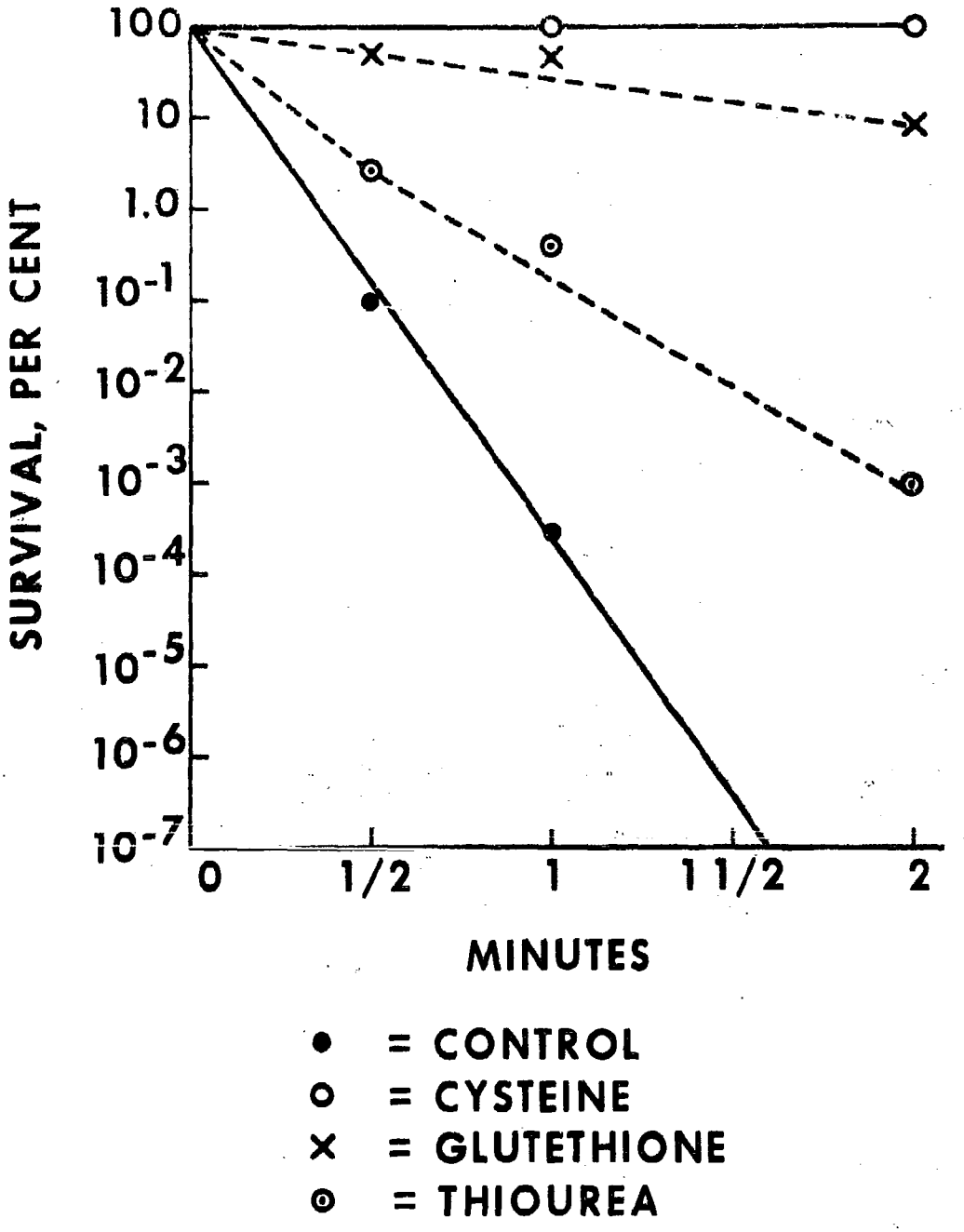

Figure 1. Inactivation of photosensitized EEE virus in the presence of reducing compounds.

The action of acridine orange on isolated infectious ribonucleic acid (IRNA) was studied by mixing infectious RNA from EEE virus with 0.25 μg/ml of acridine orange and then exposing the mixture to visible light. As shown in Table 6, control IRNA was not affected by light but combining it with acridine orange does render it photosensitive. Photoinactivation of photosensitized IRNA proceeds at a slower rate than that of photosensitized virus. The data also show that extracting the photosensitized IRNA with phenol destroyed the acridine orange-IRNA complex and the recovered IRNA was again resistant to photoinactivation. The photoinactivation rate of whole virus infectivity was compared with the infectivity of its IRNA isolated from it after given intervals of time. The data in Table 7 show that the photoinactivation of whole virus infectivity is much more rapid than the inactivation of its associated IRNA. These data combined with those showing the relatively slower rate of photoinactivation of IRNA suggest that the photodynamic effect of the dye on these viruses may also act upon the lipoprotein coat of the virus as well as on its nucleic acid and, in fact, may preferentially bind to the coat.

TABLE 6. PHOTODYNAMIC ACTION OF ACRIDINE ORANGE ON INFECTIOUS RIBONUCLEIC ACID (IRNA) FROM EEE VIRUS

	Exposure time, min		
	0	1	2
EEE-IRNA	9×10^5 a/	1.08×10^6	1.01×10^6
EEE-IRNA + 0.25 μg/ml acridine orange	1.4×10^6	9×10^4	3.0×10^4
EEE-IRNA + 0.25 μg/ml acridine orange extracted once with phenol	1.05×10^6	1.17×10^6	1.15×10^6

a. pfu/ml, $\log_{10}$.

TABLE 7. PHOTOINACTIVATION OF EEE VIRUS AND ITS ASSOCIATED NUCLEIC ACID

	Irradiation time, min			Total Inactivated[a]
	0	5	10	
EEE-proflavine complex	9.5[a]	6.2	5.1	4.4
Associated IRNA	4.6	3.6	3.0	1.6
EEE parent + 0.2 μg/ml proflavine	8.9	5.6	5.8	3.1
Associated IRNA	6.1	5.5	5.4	0.7

a. pfu/ml, $\log_{10}$.

In summary, the results presented here show that both EEE and VEE viruses were capable of being photosensitized by simply combining them in vitro with either proflavine or acridine orange dyes. The dye-virus complex was irreversible upon removal of excess dye.

The addition of reducing compounds, cysteine, glutathione, or thiourea, protected the photosensitized virus from being photoinactivated. Diluting the photosensitized virus in supernatant cell cultures also prevented complete photoinactivation of the virus. This fact prevented the use of photodynamic action to differentially separate parent virus from newly synthesized progeny, as has been accomplished with other viruses.

The IRNA was also photosensitized by the dyes, but the rate of photoinactivation was appreciably less than that for whole virus, suggesting the involvement of the lipoprotein coat. The dye could be removed from the IRNA-dye complex by phenol so that it was once again photoresistant.

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1. Wecker, E. 1959. The extraction of infectious virus nucleic acid with hot phenol. Virology 7:241-243.

2. Tomita, Y., and A.M. Prince. 1963. Photodynamic inactivation of arborviruses by neutral red and visible light. Proc. Soc. Expt. Biol. Med. 112:887-890.