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Photodynamic Inactivation of Arbor Viruses by Neutral Red and Visible Light. (28199)

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The interaction of photosensitized neutral red dye and virus:cell systems was pointed out by several groups of workers(1,2,3) as a result of observations of reduced efficiency of plaque formation when infected monolayers were exposed to visible light in the presence of dye.

During control studies on the effect of this variable on Japanese encephalitis virus:cell systems, it was noted that this virus was markedly sensitive to photodynamic inactivation by neutral red and visible light even when exposed in the extracellular state. This virus thus differed from poliovirus in this respect, since it had been reported recently(4) that photodynamic inactivation occurred only when poliovirus was grown in the presence of neutral red and not when dye and mature virus particles were mixed extracellularly.

It is the purpose of this report to document these findings, to demonstrate that they apply equally to both group "A" and group "B" arbor viruses, and to point out their technical importance, as well as their possible application to production of killed virus vaccines.

Materials and methods. Preparation of stock viruses. The Nakayama strain of Japanese encephalitis virus was employed at approximately the 90th mouse passage. The strain was "purified" by 3 successive plaque passages on chick embryo fibroblasts.

Stocks were prepared from the brains of moribund suckling mice by homogenization at 10% (W/V) concentration in 10% antibody-free rabbit or calf serum saline. Some stocks were prepared by inoculation of stable monkey kidney cells (MK-2 cells kindly supplied by Dr. Robert Hull, Eli Lilly Laboratories, Indianapolis, Ind.) maintained in M199 medium and 1% calf serum. Virus stocks were clarified by centrifugation at 10,000 rpm in the high speed head of the refrigerated International Centrifuge (Model PR-1) for 30 minutes.

The following arbor viruses were also used in this study:

a. Japanese encephalitis virus, M7-1017 selected strain isolated from mosquitoes in 1957 at the Medical General Laboratory (406) and subsequently selected for heat resistance (K. Fujinaga and R. K. Gershon, unpublished experiments).

b. Yellow fever virus, 17D strain, in 2nd Medical General Laboratory (406) suckling mouse brain passage (SMB-2).

c. West Nile encephalitis virus, Egypt 101 strain, passed once in adult mouse brain, then once in suckling mouse brain.

d. St. Louis encephalitis virus, Hubbard strain, SMB-8.

e. Murray Valley encephalitis virus, strain 1, passed at Medical General Laboratory (406) once in adult mouse brain, then once in suckling mouse brain.

f. Dengue virus, type I (Hawaiian strain), passed at Medical General Laboratory (406) 11 times in adult mouse brain, then 4 times in suckling mouse brain.

g. Western equine encephalomyelitis virus, North Dakota strain, passed 6 times in adult mouse brain, then once in suckling mouse brain.

h. Eastern equine encephalomyelitis virus in second Medical General Laboratory (406) suckling mouse brain passage.

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Virus diluent was composed of phosphate buffered saline (PBS)(5) containing 5% calf serum and adjusted to pH 7.8.

Neutral red, 0.25% (W/V) (Coleman and Bell Co., Norwood, Ohio) solution in demineralized water, was sterilized by autoclaving at 115°C for 10 minutes. The solution was stored in the dark at 5°C.

Application of light. Screw-capped test tubes (15 mm × 125 mm) containing 2 ml of virus suspensions with or without neutral red were exposed to two 15-watt Westinghouse fluorescent lamps (daylight type) held in a standard double lamp desk fixture. Tubes were irradiated at an angle of 5° from the surface of the bench. Bulbs were 6 inches from the bench top. Tubes were at right angles to the axis of the lamps. While not being irradiated, virus suspensions were held in lidded stainless steel jars containing ice water.

Dilution, inoculation, and addition of agar overlays were performed in dim light to minimize photodynamic inactivation.

Chick embryo fibroblasts. Chick embryo fibroblasts were prepared by trypsinization of decapitated, minced, 10- to 11-day-old chick embryos in PBS without divalent cations. Two-ounce, rubber-stoppered prescription bottles were inoculated with 2.8×10^7 cells in 8.0 ml of modified Gey's solution(6) containing 0.04 M tris buffer, pH 7.2, and 3.5% inactivated antibody-free calf serum. The cell monolayers were used for plaque assay after 2 days of incubation at 35°C.

Plaque assay. Inocula of 0.2 ml were added to 2 or 3 assay monolayers for plaque formation. Bottles were then held in the dark for 2 hours at 35°C to permit near maximal adsorption of virus. Overlay medium was composed of Gey's solution containing 0.04 M tris buffer, pH 7.5, 0.3% tryptose phosphate broth, 0.5% lactalbumin hydrolysate, 4% inactivated calf serum, 0.005% neutral red, and 1% Difco Noble agar. Media contained 50 units of kanamycin, 100 μg streptomycin and 100 units of mycostatin per milliliter. Plaque assay bottles were incubated at 35°C and were counted when plaque count became stable.

Results. Effect of neutral red on suspensions of Japanese encephalitis virus. It will be seen in Fig. 1 that when virus was sus-

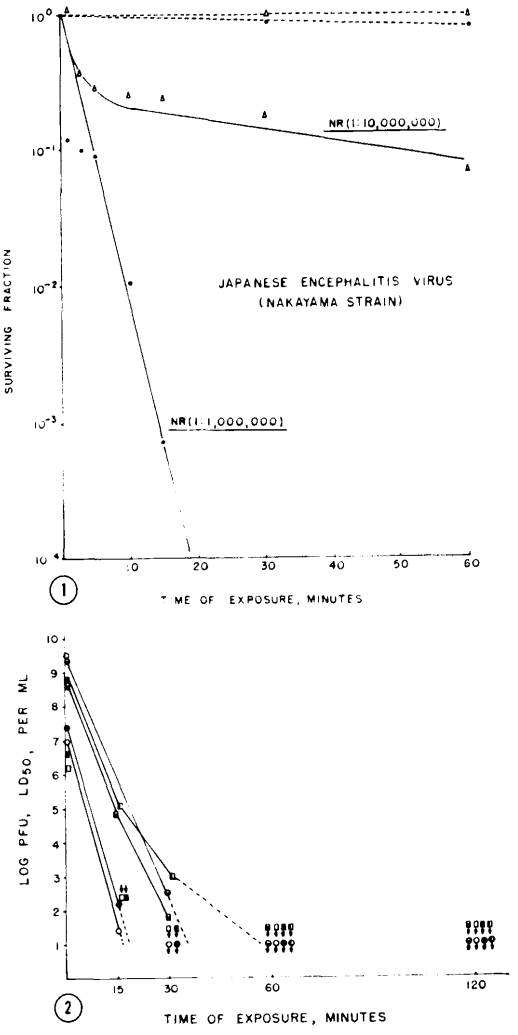

FIG. 1. Inactivation of Japanese encephalitis virus, Nakayama strain, by neutral red and visible light. Solid lines refer to tubes exposed to visible light (see *Materials and methods*), dotted lines refer to control tubes containing neutral red but maintained in darkness. The concentration of neutral red was 1:1,000,000 (circles) or 1:10,000,000 (triangles).

FIG. 2. Inactivation of high titer stocks of Japanese encephalitis virus by neutral red and visible light. Undiluted 10% suckling mouse brain stocks (half filled circles or squares), or stocks prepared on MK-2 cells, *in vitro* (filled or empty circles or squares) were mixed with an equal volume of PBS containing 1:50,000 neutral red. Assays were carried out after various times of exposure to light by plaque formation (circles), or by inoculation of 4-wk-old mice intracerebrally (squares). Stocks employed were as follows: M7-1017 selected strain mouse brain stock (circles and squares half filled on bottom); M7-1017, MK-2 stock (open circles and squares); Nakayama strain, mouse brain stock (circles and squares half filled on left side; Nakayama strain, MK-2 stock (closed circles and squares).

TABLE I. Effect of Photodynamic Action of Neutral Red on Other Arbor Viruses.

Virus	Exposure to visible light*	Concentration of neutral red	Titer (PFU/ml) at 0 min	60 min	120 min
Eastern equine Encephalitis	—	0	7.5×10^4	—	4.3×10^4
	+	"	"	6.2×10^4	4.5×10^4
	—	1:240,000	"	4.4×10^4	4.4×10^4
	+	"	"	0	0
Western equine Encephalitis	—	0	3.6×10^4	—	2.8×10^4
	+	"	"	2.5×10^4	2.6×10^4
	—	1:240,000	"	"	2.3×10^4
	+	"	"	0	0
West Nile Encephalitis	—	0	3.6×10^2	4.4×10^2	—
	+	"	"	4.6×10^2	—
	—	1:100,000	4.3×10^2	3.4×10^2	—
	+	"	"	0	—
Yellow Fever	—	0	2.6×10^2	2.7×10^2	—
	+	"	"	2.3×10^2	—
	—	1:100,000	1.5×10^2	1.9×10^2	—
	+	"	"	0	—
St. Louis Encephalitis	—	0	1.2×10^2	1.7×10^2	—
	+	"	"	1.4×10^2	—
	—	1:100,000	1.6×10^2	—	—
	+	"	"	0	—
Murray Valley Encephalitis	—	0	1.0×10^3	8.8×10^2	—
	+	"	"	9.1×10^2	—
	—	1:100,000	"	8.4×10^2	—
	+	"	"	0	—

* Tubes exposed for 30 min at 27.8°C.

pended in a concentration of 1:1,000,000 neutral red, rapid exponential inactivation was observed. The effect was markedly reduced with neutral red at a concentration of 1:10,000,000.

Control suspensions containing neutral red but not exposed to light were not significantly inactivated, indicating that the dye owed its virucidal properties to photodynamic action.

The first order characteristics of the inactivation kinetics would appear to suggest that one molecule of dye is sufficient to inactivate a virus particle.

Rapid viral inactivation by low concentrations of a nontoxic dye appeared to offer the possibility that this method might be used for production of killed viral vaccines. It was thus of interest to determine whether high titer stocks could be completely inactivated by this method.

Fig. 2 summarizes an experiment in which undiluted suckling mouse brain and MK-2 tissue culture stocks were exposed to visible light in the presence of 1:100,000 neutral red. It was found that virus became nondetectable by both plaque assay, and by inoculation of 4-week-old mice intracerebrally, after between 30 and 60 minutes of exposure to light. In the case of the mouse brain stocks this represented more than 8 log units of inactivation.

Effect of neutral red on other arbor viruses. Tables I and II summarize experiments designed to determine the susceptibility of other arbor viruses to this form of inactivation. All viruses tested were readily inactivated by visible light in presence of neutral red and were not affected by either light or neutral red alone.

Discussion. It has been found in these ex-

TABLE II. Effect of Neutral Red and Visible Light on Infectivity of Dengue Virus for Mice.

Exposure to* visible light	Incorporation of neutral red (concentration)	Virus† dilution	Rate death	% mortality
—	1:100,000	10^{-2}	7/9	77.8
		10^{-3}	4/9	44.4
—	None	10^{-2}	7/9	77.8
		10^{-3}	3/10	30.0
+	1:100,000	10^{-2}	0/9	0
		10^{-3}	0/10	"
+	None	10^{-2}	6/9	66.7
		10^{-3}	2/8	25.0

* Exposure at 27.8°C for 30 min.

† Diluent: PBS with 5% normal rabbit serum, pH 7.8. Inoculum size: .03 ml/mouse.

periments that Group "A" and Group "B" arbor viruses were highly susceptible to rapid inactivation by the combined action of neutral red and visible light. The arbor viruses thus resemble in this respect bacteriophages, adenoviruses, "B" virus (herpes symiae), and vaccinia, all of which have been found(7) sensitive to photodynamic inactivation by vital dyes of the acridine series of which neutral red is a member. These viruses differ in this respect from enteroviruses which resist this form of inactivation(4,8).

It has been postulated by Hiatt that the differential susceptibility of viruses to photodynamic inactivation by vital dyes reflects difference in permeability of the outer virus coat, or capsid, to penetration of dye(7). This hypothesis is supported by the finding of Growther and Melnick(4) that enteroviruses become susceptible to photodynamic inactivation with vital dyes only when grown in the presence of these dyes so that dye-nucleic acid complexes may be formed without the necessity for capsid penetration. This hypothesis is also supported by the first order inactivation kinetics, which suggest that genetic rather than capsid damage is produced.

Conversely, the lack of photodynamic action on enteroviruses, in the light of the above hypothesis, would suggest that the acridine dyes, at least at the low concentrations employed, do not cause significant damage to proteins.

If these interpretations are correct, photodynamic inactivation in the presence of neutral red may offer an ideal method for production of killed virus vaccines from those agents sensitive to this form of inactivation. This method may result in little or no destruction of antigenic activity.

Preliminary experiments reported herein indicated that more than 8 log units of inactivation may occur without evidence of the presence of a fraction of virus resistant to this form of inactivation. Due to the possibility that an interference phenomenon masked the survival of a minor fraction of resistant virus, further experiments should be carried out before it can be safely concluded that complete inactivation of all viable virus was achieved when stocks of high virus concentration were inactivated.

The susceptibility of viruses to this form of inactivation may be expected to pose practical difficulties in experiments carried out *in vitro* in the presence of neutral red, *e.g.*, conventional plaque assays.

In the present system, however, it was found that the sensitivity of the plaque assay with Japanese encephalitis virus was reduced only about 2-fold when neutral red was present throughout the assay, and when standard laboratory lighting conditions were employed (N. Hashimoto and Y. Tomita, unpublished experiments). However, it may be expected that the concentration of viable virus which can be harvested from single plaques in clone isolation experiments will be markedly influenced by this variable.

Summary. Six Group "B" and 2 Group "A" arbor viruses were found to be highly susceptible to inactivation by neutral red in the presence but not in the absence of visible light.

Inactivation kinetics with Japanese encephalitis virus were first order and proceeded for more than 8 log units when high titer stocks were investigated.

The possible application of this method of inactivation to the production of killed virus vaccines was discussed.

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