Inactivation of Foot-and-Mouth Disease Virus by Interaction of Dye and Visible Light

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ABSTRACT

FELLOWES, O. N. (Plum Island Animal Disease Laboratory, Greenport, N.Y.). Inactivation of foot-and-mouth disease virus by interaction of dye and visible light. Appl. Microbiol. 14:86-91. 1966.—The inactivation of foot-and-mouth disease virus was studied by means of the interaction of neutral red, Toluidine Blue, and methylene blue with visible light. The virus, Type A, strain 1. CANEFA of Argentine origin, was grown in tissue culture and tested in the crude and clarified state. Virus and dye were mixed and incubated together at 4°C for 45 min in the dark, or were mixed and immediately exposed to the visible light source without prior incubation together. Mixtures of crude virus and dye, under any of the experimental conditions used, did not inactivate more than 1 to 2 logs of viral infectivity when held in the dark or when exposed to light during a period of 45 min. Complete inactivation of virus was achieved when clarified virus and dye were mixed and immediately exposed to the visible light source for 15 min. Prior incubation of clarified virus and dye permitted inactivation by methylene blue only, whereas no incubation prior to exposure resulted in three of the dyes contributing to inactivation. A concentration of 6 μg of neutral red, Toluidine Blue, methylene blue, and crystal violet was used per milliliter of virus suspension. Crystal violet was not a good viral inactivator under the conditions of the experimentation. Inactive virus induced the formation of neutralizing antibodies in adult chickens and mice. The antibody titer stimulated by the antigen treated with methylene blue and visible light was probably significant.

The phenomenon of photosensitization of a biological system through the agency of a chemical and visible light, so that photochemical reactions may take place, was first described by Raab (11). Perdrau and Todd (10) made an unsuccessful attempt to inactivate canine distemper virus in tissue suspension by the interaction of methylene blue and visible light. The virus in filtrates or in fluids without cells could be inactivated photochemically with retention of antigenicity. Galloway (4) found foot-and-mouth disease virus (FMDV) resistant to the action of visible light and neutral red, eosin, auramine, pyronine, Toluidine Blue, erythrosin A, nigrosin, and naphthol green. The virus was inactivated by visible light and protoporphyrin, rivanol, and acriflavine. Hiatt (6) feels that the viral nucleic acid is the site of the photodynamic reaction which inactivates. After testing a number of viruses for inactivation with Toluidine Blue and visible light, this author suggested that, as a class, the deoxyribonucleic acid (DNA) viruses are susceptible and ribonucleic acid (RNA) viruses are resistant to such treatment. In an additional study, Hiatt et al. (7) treated 11 different viruses with Toluidine Blue to obtain rates of inactivation and found a wide range of variation corresponding more or less with accepted taxonomic groupings. Orlob (9) has examined photodynamic inactivation of plant viruses using neutral red or acridine orange. Prior incubation of virus and dye was required for inactivation, apparently allowing the dye to penetrate and reach the RNA core. Cytochemical and fluorescence tests showed dye attachment to RNA only in fixed (preincubated) preparations. Wallis and Melnick (14) found that, if mature poliovirus was freed from extraneous suspended material and organic buffers, dialyzed, and diluted 100-fold at pH 8.0, the virus could be photodynamically inactivated with neutral red, methylene blue, Toluidine Blue, and proflavin. All of these findings suggested an investigation in which FMDV would be inactivated by the photodynamic approach while striving to retain the
maximal antigenicity in the treated virus for immunogenic studies in adult mice and chickens.

**Materials and Methods**

**Virus.** The virus used in this study was type A, strain 1, CANEFA, in its third tissue culture passage. The virus was grown in calf kidney cell culture in Povitsky bottles. Both crude and clarified virus were used for the test preparations. For clarified virus, the maintenance fluid did not contain phenol red or other dye when applied to the infected cells. Centrifugation of the cultivated virus at 10,000 × g for 30 min in a Spinco centrifuge with a no. 21 rotor, in which each tube held 75 ml, yielded suspensions relatively free from large particles. Removal of debris and large particles dropped the virus concentration about 1 log in infectivity.

**Dyes.** The dyes tested for inactivation were neutral red, Toluidine blue, methylene blue, and crystal violet. All dyes were certified as to their dye content by the Biological Stain Commission. The concentration used was 6 μg of dye per ml of virus suspension.

**Visible light source.** The apparatus used was composed of two 100-w incandescent light bulbs placed 6 inches apart on a platform with a 600-ml beaker centered midway between them. Tubes, each containing 7 ml of the virus-dye mixture, were exposed to the light and then tightly stoppered and submerged in a beaker filled with cold water. Virus-dye mixtures were exposed to the visible light source for periods of 15, 30, and 45 min. All sources of light other than the incandescent bulbs were excluded from the virus.

**Tubes.** Pyrex tubes (13 by 100 mm) were selected for uniform diameter and wall thickness by use of a vernier caliper. The selected tubes were then checked for transmission of light at a wavelength of 625 μ, by use of Hanks’ balanced salt solution (BSS) with serum, but without dye, as a blank fill in a volume of 7 ml. By my means of a grid over the mouth of a beaker, three tubes and a thermometer were immersed in the contents of the beaker. Temperature of water in the beaker did not rise above 15°C.

**Procedure.** Crude virus with a content of 10^6.5 to 10^7 plaque-forming units (PFU)/ml and clarified virus at 10^5.5 PFU/ml were mixed with the respective dyes to make a concentration of 6 μg/ml of virus. The mixtures were adjusted to pH 7.5 with 1 N NaOH, since the virus was sensitive to pH 6.5 and lower. The following preparations were made and treated as described. (i) Crude virus and dye were mixed, incubated together for 45 min at 4°C in the dark, and exposed to visible light for 15, 30, and 45 min. (ii) Crude virus and dye were mixed and immediately exposed to visible light as in (i). (iii) Clarified virus and dye were mixed and incubated together for 45 min at 4°C before exposure to visible light as in (i). (iv) Clarified virus and dye were mixed and immediately exposed to visible light as in (i). (v) Original virus control was held at 4°C for period of test procedure. (vi) Original virus without dye was exposed to the light source for a period of 45 min as a control.

**Infectivity test.** The samples, after treatment, and controls were serially diluted and assayed for PFU on monolayers of calf kidney cell cultures. Three 4-oz (113.4 g) prescription bottles of cells were used per dilution. The virus was incubated on the cell sheet for 30 min at 37°C before being covered with an overlay composed of Hank’s BSS, neutral red, bovine serum, and agar. The PFU per milliliter were calculated from the readings obtained after 48 to 60 hr of additional incubation at 37°C.

**Immunogenicity tests.** Inactive virus was prepared by mixing methylene blue, neutral red, or Toluidine Blue with clarified virus and immediately exposing the mixture to visible light for 15 min. Single-comb, white Leghorn chickens, 6 months to 1 year in age, were given intravenous injections of 2 ml of the treated virus preparation diluted 1:10. Five birds were used per sample, and, 1 week later, the fowl were bled, and the serum was obtained and frozen (3). Adult mice, Rockefeller Institute H strain, weighing 18 to 20 g each, were placed in groups of 20; each mouse received two 0.25-ml intramuscular doses, given a week apart, of the treated viral preparation diluted 1:10. The mice were bled, in groups of 20, 3 weeks after the administration of the first dose of trial antigen, and the serum was pooled and frozen. Active virus, diluted 1:10 or undiluted, was inoculated into chickens and mice as a control procedure.

A neutralization test was finally chosen which involved varying dilutions of virus and a fixed dilution of the various sera with appropriate controls (13). Suckling mice, 5 to 7 days old, were inoculated intraperitoneally with 0.05 ml of the virus-antiserum mixture, which had been incubated for 1 hr at 37°C; 10 mice per dilution were used. Type A, strain 1. CANEFA, in its third tissue culture passage, was used as the test virus. The end point of the test was the difference between the LD₅₀ value of virus plus normal serum and the LD₅₀ value of virus plus experimental serum resulting in a neutralization index.

**Results**

The experimental results discussed as follows were the mean of three tests of three different samples investigated at different times.

Samples of crude virus incubated with each of four dyes for 45 min at 4°C in the dark and then exposed to visible light for an additional 15, 30, and 45 min showed some inactivation (Fig. 1). The action of the dye alone reduced the virus infectivity by a maximum of 0.5 log in 45 min in the dark. The viral infectivity was reduced an additional 1 log when crude virus was treated with dye for 45 min in the dark and then exposed to light. Virus alone exposed to light for 45 min experienced little loss of infectivity.

Figure 2 shows the results of similar treatment of crude virus and dye by exposure to visible light without prior incubation of the mixture. The loss of viral infectivity by dye action in the dark control and after exposure of dye-treated virus to visible light was about the same as in Fig. 1: 0.5 log and 1 log, respectively, a total loss of about 1.5 logs.
Clarified virus when incubated with the various dyes in the dark for 45 min experienced a maximal loss of 3.5 logs in infectivity (Fig. 3). However, when preincubated mixtures of virus and dye were exposed to visible light, the mixture containing methylene blue lost all its remaining infectivity in 15 min. The virus treated with Toluidine Blue and neutral red lost a maximum of 3 logs of activity in 45 min in the dark, but still retained 1 log of infectivity after a further exposure to visible light for 45 min.

Clarified virus, mixed with the various dyes and immediately exposed to visible light, was inactivated in 15 min in the mixtures containing methylene blue, neutral red, or Toluidine Blue (Fig. 4). No active virus was detected by tissue culture methods in the clarified virus-dye mixtures exposed immediately to visible light. Figure 4 also shows that a maximum of 3 logs of viral activity was lost in the dye controls held for 45 min in the dark. Since no prior incubation of mixtures was required to inactivate the virus in 15 min, it can be said that the interaction of dye and visible light was responsible. Clarified virus alone exposed to visible light for 45 min experienced little loss in activity.

Crystal violet did not show any particular ability to inactivate FMDV under the conditions of these experiments.

The immunogenicity of the various inactive preparations, as compared with that of active virus in chickens and mice, is shown in Table 1. The virus preparations were exposed to visible light without preincubation of the dye and virus mixture. All three dye-virus preparations induced neutralizing antibody formation in both adult mice and chickens. The level of antibody produced by the methylene blue-virus mixture was the highest, and is probably significant.

**DISCUSSION**

FMDV was resistant to inactivation by exposure to visible light, as shown by control virus exposed to visible light in Fig. 4 and by experimental work done by Skinner and Bradish (12). It was expected that, if a dye and visible light were to inactivate the virus by photodynamic action, the dye and virus would have to be incu-
bated together for a period of time before exposure to light. Such was not the case with FMDV when utilized in a clarified state with the dye concentration and light source used in this study. The elimination of light-absorbing dye from the tissue culture maintenance medium and most of the large particle debris from the final virus suspension may have aided the photochemical reaction.

Crystal violet is capable under certain conditions of inactivation of FMDV in the whole blood from viremic animals (5). It has been shown by Anan'ev and Blinova (1) that Russian tick-borne encephalitis virus, treated with a 1:25,000 concentration of methylene blue in saline, can be inactivated in 10 min by exposure to the visible light from a 96-w incandescent bulb at a distance of 20 cm. When the virus was suspended in serum, inactivation under the same conditions took place in 3 hr.

The conditions of test in a single trial of the inactivation experiments did not yield sufficient inactive virus to perform infectivity tests and to permit immunization of groups of chickens and mice with undiluted material. Therefore, the antibody level shown in Table 1 was produced by the inoculation of a 1:10 dilution of the treated virus into chickens and mice and was the mean of three replicates performed at different times. Control preparations of active virus, undiluted and diluted 1:10, were inoculated into test animals to determine the levels of antibody produced by these untreated antigens. The antibody titer induced by exposure to visible light of methylene blue-treated virus more nearly approximated that induced by active virus diluted 1:10 than did any other similarly diluted combination of inactivant and virus acting as antigen. The virus appeared to be harmed or degraded in its antigenic capacity by treatment with neutral red or Toluidine Blue.

Type A, strain 1, CANEFA, had approximately the same titer in logs per milliliter in cattle tongue, suckling mice, and tissue culture as measured by ID_{50}, LD_{50}, and PFU, respectively. Theoretically, residual active virus between log 1.0 and log 0.0 cannot be regularly demonstrated in any test system known. It is difficult, if not impossible, to determine whether the antibody response demonstrated with treated virus is due to

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**Fig. 3.** Inactivation of clarified foot-and-mouth disease virus by various dyes and visible light. Virus and dye were incubated together at 4°C for 45 min prior to exposure to light. Symbols: OVC, original virus control; LVC, light virus control; CV, crystal violet; NR, neutral red; TB, Toluidine Blue; MB, methylene blue; DVC, dark virus control not exposed to light. Broken lines indicate no virus was found in undiluted treated preparation.

**Fig. 4.** Inactivation of clarified foot-and-mouth disease virus by various dyes and visible light. Virus and dye were not incubated together prior to exposure to light. Symbols: OVC, original virus control; LVC, light virus control exposed to light only; DVC, dark virus control not exposed to light; CV, crystal violet; NR, neutral red; TB, Toluidine Blue; MB, methylene blue. Broken line indicates no virus was found in undiluted treated preparations.
inactivated virus or to residual active virus in a nonproliferating host, or, perhaps, to a mixture of both.

The significance of the level of antibody produced by the inactive antigens in this study as influenced by the normal controls of virus, chicken serum, and mouse serum may be of interest, since these values control the level of the neutralization index and its validity. A standard deviation was calculated for 11 pairs of normal chicken and mouse serum controls compared with their respective virus control. The deviation of the mean for the virus control was 0.17 log; for the chicken serum control, 0.20 log; and for the mouse serum control, 0.56 log. Of the sample means of each series ± one standard deviation, the virus control means fell within the calculated range in 9 of 11 instances, the chicken serum means had 9 of 11 observations within the range, and the mouse serum means were 11 of 11 within the range. A calculation of the confidence limits showed that 8 of 11 virus control means fell in the 99% limit, whereas, in both the chicken and mouse serum controls, 9 of 11 of the observed means came in the 99% range. A "t" test for significance of the relationship of the sample means of the normal chicken and normal mouse serum series to the means of the virus control gave values of 2.4 and 2.5 with 10° of freedom. This places the significance of any difference at the 5% level or 1 in 20 by chance. The 5% level is not considered significant; therefore, the means are related and from a normal population.

It has been demonstrated that normal cattle sera used in the constant serum-variable virus dilution technique in the neutralization test may vary in their ability to neutralize virus from 0.1 log to as high as 2.6 log of activity (1a, 2). It was interesting to note that Nottebohm et al. (8) used adult chickens along with cattle to test FMDV vaccine. It was the opinion of these workers that any neutralization index of 2 or above was indicative of significant antibody production in the chicken.

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LITERATURE CITED


the photodynamic action of toluidine blue. J. Immunol. 84:480-484.


