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Adjuvant Effects of Tilorone Hydrochloride (Analog 11,567) with Inactivated Venezuelan Equine Encephalomyelitis Virus Vaccine (41055)[1,2]

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Abstract. Tilorone analog 11,567 (BDD) was demonstrated to immunopotentiate Formalin-inactivated Venezuelan equine encephalomyelitis (VEE) TC-83 vaccine in mice and monkeys. The dosage of BDD required for adjuvant effects was different in mice and monkeys. In monkeys, primary and secondary antibody responses were increased (4- and 12-fold greater titers, respectively) when 10 mg/kg of BDD was given with VEE vaccine; however, 100 mg/kg of BDD had no adjuvant effect. Mice given 62 or 250 mg/kg of BDD with undiluted vaccine developed 3- and 4-fold greater antibody titers, respectively, than vaccine controls. The immunopotentiation effect of BDD was most dramatically demonstrated when graded doses (7 to 500 mg/kg) were given with a marginally antigenic dose of VEE vaccine (diluted 1:4). The BDD–vaccine combination induced antibody production, while vaccine administered without adjuvant did not. Antibody titers were significantly higher when 125 ($P < 0.001$) or 250 ($P < 0.05$) mg/kg of BDD were given with the diluted vaccine. Administration of 500 mg/kg of BDD did not increase antibody titers. Survival of mice challenged 14 days after vaccination was significantly greater ($P < 0.05$) in groups administered undiluted VEE vaccine containing BDD than in vaccine controls groups; although survival was not significantly greater than in vaccine groups given Freund's complete (FCA) or incomplete (FIA) adjuvant. However, when BDD was used with marginally antigenic doses of vaccine, survival was significantly greater ($P < 0.05$) than when FCA or FIA were used as adjuvants. The minimal dose of BDD (125 mg/kg) required to significantly potentiate antibody response to diluted vaccine was near the safety limits as ≥250 mg/kg induced skin lesions in mice.

Tilorone hydrochloride, a low-molecular-weight, water-soluble, dihydrochloride salt of 2,7-bis[2-(diethylamino)ethoxy]-fluorene-9-one is an active interferon inducer and antiviral drug when administered to rodents by oral and parenteral routes (1–4). However, the drug does not induce endogenous interferon in humans or monkeys (5, 6). Tilorone has been reported to have diverse effects on host immune defense mechanisms including antiviral (3, 7), antitumor (8–11), phagocyte stimulation (10, 12, 13), and adjuvant (14) activities. Tilorone is capable of selectively enhancing humoral immunity while at the same time suppressing cellular immune responses in rodents (15). This latter effect may be due to drug-induced changes in host lymphocyte populations (16). The effects of tilorone on cell-mediated and humoral immune responses have been reviewed by Megel *et al.* (17). Tilorone analog 11,567 {2,8-bis-

[1] In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care. The views of the authors do not purport to reflect the positions of the Department of the Army or the Department of Defense.

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[2-(dimethylamino)acetyl]-dibenzofuran dihydrochloride} (BDD) used in this study as biological activities that are similar to those of the parent compound but has the advantage of being less toxic (7). This study was conducted to determine if tilorone analog 11,567 would potentiate the immune response in mice and monkeys given Formalin-inactivated Venezuelan equine encephalomyelitis (VEE) virus vaccine.

Materials and Methods. *Animals.* Outbred male Swiss (CD-1) mice, weighing 19 to 21 g, were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Massachusetts. Mice, housed 5 to 10 per cage, were provided food (Wayne Rodent Diet, Allied Mills, Inc., Chicago, Ill.) and water *ad libitum*. Laboratory conditioned, 3- to 5-kg rhesus monkeys (*Macaca mulatta*) of either sex, seronegative for VEE virus were obtained from the Institute's primate colony. Monkeys were housed in individual cages, fed commercial monkey chow twice daily (Monkey Diet, Ralston Purina Co., St. Louis, Mo.), and provided water *ad libitum*.

Vaccine. Formalin-inactivated VEE vaccine (strain TC-83, Lot E-96) (18) was used either undiluted or diluted 1:4 in sterile saline (previously determined to be a marginally antigenic dose). All vaccinations were administered sc.

Adjuvants. Tilorone hydrochloride analog 11,567 (BDD), was kindly supplied by Merrell-National Laboratories, Cincinnati, Ohio. BDD was dissolved in sterile saline and mixed with vaccine or an equivalent volume of sterile saline just before sc inoculation. Freund's complete and incomplete adjuvants (FCA and FIA, Difco Laboratories, Detroit, Mich.) were used as adjuvant controls. Aqueous VEE vaccine was added to FCA or FIA in a 1:1 (v/v) ratio and emulsified before inoculation.

Challenge virus. Trinidad donkey strain of VEE virus was used in all challenge studies (19). Viral inocula were diluted in Hanks' balanced salt solution containing 1% heat-inactivated (56° for 30 min) fetal bovine serum. Mice were challenged by ip injection of 0.3 ml of VEE viral suspension containing 250 to 2400 mouse median ip lethal doses ($MIPLD_{50}$) as described in individual experiments. The virus challenge inoculum was titrated by ip injection of serial 10-fold dilutions into nonvaccinated mice; the $MIPLD_{50}$ was calculated as previously described (20).

Serologic test. Titrations for VEE virus plaque-reduction neutralizing (PRN) antibody were performed as described (21). Titers were expressed as the reciprocal of the greatest serum dilution giving 80% plaque reduction (PRN_{80}). Titers below 8 were assigned a value of 4 for calculation of geometric mean (GM) titers.

Experimental design. The adjuvant dose effect of BDD was determined by sc inoculation of groups of 20 mice with graded doses (7 to 500 mg/kg) of BDD combined with 0.25 ml of VEE vaccine diluted 1:4. Groups of control mice were inoculated with vaccine, saline, or BDD and saline. On Day 14 postvaccination, 4 mice from each group were bled and VEE antibody titers were determined. The remaining 16 mice were challenged ip with 1000 $MIPLD_{50}$ of virus and percent survivors were calculated on Day 21 postchallenge.

For determination of antibody response and protection against challenge, groups of 25 mice were inoculated sc with 0.25 ml of undiluted vaccine combined with 62 or 250 mg/kg of BDD. Control groups of mice were inoculated sc with vaccine, saline, or BDD and saline. Adjuvant control groups were inoculated sc with either vaccine or saline emulsified in an equal volume of FCA or FIA. On Day 14 postvaccination, 5 mice from each group were bled and VEE antibody titers were determined. The remaining 20 mice were challenged with 250 $MIPLD_{50}$ of VEE virus. Mice were observed daily and percentage survivors were calculated on Day 21 postchallenge.

A second determination of antibody response and immune protection against challenge was conducted in mice as above, except that the vaccine was diluted 1:4 before combination with BDD; VEE viral challenge was increased to 2400 $MIPLD_{50}$.

The adjuvant effect of BDD was assessed in rhesus monkeys by inoculation of 3 monkeys in each of 3 groups with 2 doses of undiluted vaccine (0.5 ml) at 28-day intervals using the following concentrations of

BDD: groups 1, 10 mg/kg; group 2, 100 mg/kg; and group 3, saline control. After inoculation, venous blood samples were collected at prescribed intervals for determination of VEE antibody titers.

Results. Adjuvant effects of BDD combined with VEE vaccine are evident in antibody response and challenge survival data detailed in Table I. Survival to challenge was significantly ($P < 0.05$) increased in six of seven groups given vaccine containing 7 to 500 mg/kg of BDD when compared to groups administered vaccine without adjuvant. Reciprocal PRN_{80} antibody titers ≥ 8 were present at the time of challenge in individual animals from all groups of mice that received vaccine containing BDD. Reciprocal GM antibody titers were significantly greater for groups of mice given vaccine containing 125 ($P < 0.001$) and 250 mg/kg ($P < 0.05$) of BDD than for the vaccine control group. Minimal adverse signs of adjuvant toxicity or reactogenicity occurred in vaccine groups administered ≤ 125 mg/kg of BDD. However, mice inoculated with ≥ 250 mg/kg of BDD developed localized skin lesions at the inoculation site. Significant systemic toxicity was not demonstrated. None of the control mice given 250 or 500 mg/kg of BDD died before challenge.

Survival after challenge with 250 $MIPLD_{50}$ of VEE virus on Day 14 postvaccination was significantly greater ($P < 0.05$) in mice administered VEE vaccine containing BDD than in mice given VEE vaccine without adjuvant (Table II). Furthermore, PRN_{80} antibody titers were three- to fourfold greater in mice given VEE vaccine containing BDD than in vaccine control mice. Antibody titers and survival for FCA or FIA vaccine groups were not significantly different from the vaccine control group.

After vaccination with a marginally antigenic dose of vaccine (diluted 1:4), survival after challenge on Day 14 postvaccination with 2400 $MIPLD_{50}$ of VEE virus was significantly higher ($P < 0.05$) in mice administered vaccine containing BDD than in mice given vaccine containing control adjuvants (FCA or FIA) or in vaccine controls (Table III). Furthermore, antibody titers ≥ 8 were detected only in mice that received vaccine containing BDD.

Monkeys administered two doses of undiluted vaccine with 10 mg/kg of BDD developed primary and secondary GM anti-

TABLE I. ADJUVANT EFFECT OF GRADED DOSES OF TILORONE ANALOG 11,567 (BDD) WITH FORMALIN-INACTIVATED VENEZUELAN EQUINE ENCEPHALOMYELITIS (VEE) VIRUS VACCINE IN MICE

Treatment	Dose of BDD (mg/kg)	Reciprocal geometric mean Antibody titer (PRN_{80}) Day 14 ($n = 4$)	Percentage survival[a] Day 35 ($n = 16$)
Vaccine[b] + BDD	7	12	56
	15	12	69[f]
	31	8	94[g]
	62	14	94[g]
	125	54[c]	100[g]
	250	26[d]	100[g]
	500	5	88[g]
Vaccine controls	0	—[e]	19
BDD controls	250	—	0
	500	—	0
Saline controls	0	—	0

[a] Mice challenged ip on postvaccination Day 14 with 1000 $MIPLD_{50}$ of VEE virus.
[b] Mice administered 0.25 ml of vaccine diluted 1:4 (sc).
[c] $P < 0.001$ compared to vaccine controls by Student's t test.
[d] $P < 0.05$ compared to vaccine controls by Student's t test.
[e] — = antibody not detectable (<8).
[f] $P < 0.05$ compared to vaccine controls by χ^2 analysis with Yates' correction.
[g] $P < 0.001$ compared to vaccine controls by χ^2 analysis.

TABLE II. ADJUVANT EFFECT OF TILORONE ANALOG 11,567 (BDD) WITH FORMALIN-INACTIVATED VENEZUELAN EQUINE ENCEPHALOMYELITIS VIRUS VACCINE IN MICE

Treatment	Dose of BDD (mg/kg)	Reciprocal geometric mean antibody titer (PRN_{80}) Day 14 (n = 5)	Percentage survival[a] Day 35 (n = 20)
Vaccine[b] + BDD	62	64	100[c]
	250	46	100[c]
Saline + BDD	250	—[d]	0
Vaccine + FCA (1:1)	0	12	90
Saline + FCA (1:1)	0	—	35
Vaccine + FIA (1:1)	0	10	40
Saline + FIA (1:1)	0	—	0
Vaccine control	0	16	70
Saline control	0	—	0

[a] Mice challenged ip on postvaccination day 14 with 250 $MIPLD_{50}$ of VEE virus.
[b] Mice administered 0.25 ml of undiluted vaccine sc.
[c] $P < 0.05$ compared to vaccine controls χ^2 analysis with Yates' correction.
[d] — = antibody not detectable (<8).

body titers which were 4- and 12-fold greater, respectively, than titers in monkeys given two doses of undiluted vaccine without adjuvant (Fig. 1). In contrast, neither the primary nor secondary antibody response of monkeys administered vaccine with 100 mg/kg of BDD was enhanced compared to the antibody response in vaccine controls.

Discussion. The mechanism for the broad-spectrum antiviral activity of oral tilorone was originally attributed to induction of interferon (1, 3). However, Giron *et al.* (22) reported that antiviral activity did not always correlate with interferon induction. It is now known that tilorone has a multiplicity of effects on the immune system. Tilorone suppresses a variety of cell-mediated immune responses in rodents, including paralysis after experimental allergic encephalomyelitis, paw edema associated with adjuvant arthritis, tuberculin skin reaction, and local graft-versus-host response (17). This effect is mediated by a specific effect on T lymphocytes and accompanied by enhancement of B lympho-

TABLE III. ADJUVANT EFFECT OF TILORONE ANALOG 11,567 (BDD) WITH A MARGINALLY ANTIGENIC DOSE OF FORMALIN-INACTIVATED VENEZUELAN EQUINE ENCEPHALOMYELITIS (VEE) VIRUS VACCINE IN MICE

Treatment	Dose of BDD (mg/kg)	Reciprocal geometric mean Antibody titer (PRN_{80}) Day 14 (n = 5)	Percentage survival[a] Day 35 (n = 20)
Vaccine[b] + BDD	62	10	70[c]
	250	12	63[c] (n = 19)
Saline + BDD	250	—[d]	0
Vaccine + FCA (1:1)	0	—	15
Saline + FCA (1:1)	0	—	0
Vaccine + FIA (1:1)	0	—	5
Saline + FIA (1:1)	0	—	5
Vaccine control	0	—	0
Saline control	0	—	0

[a] Mice challenged ip on postvaccination Day 14 with 2400 $MIPLD_{50}$ of VEE virus.
[b] Mice administered 0.25 ml of vaccine diluted 1:4 (sc).
[c] $P < 0.001$ compared to vaccine controls by χ^2 analysis with Yates' correction.
[d] — = antibody not detectable (<8).

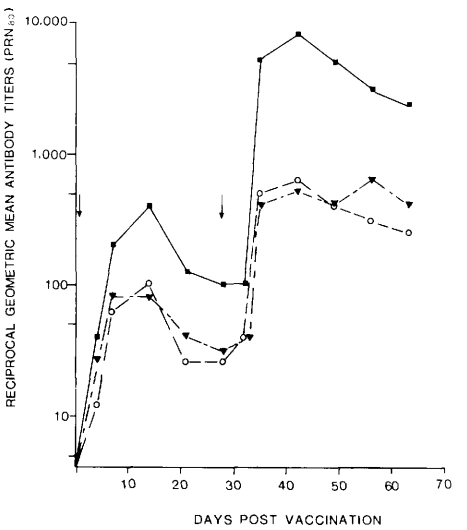

FIG. 1. Reciprocal geometric mean antibody titers (PRN_{80}) in monkeys after two doses (arrows) of Formalin-inactivated Venezuelan equine encephalomyelitis (VEE) virus vaccine alone (○) and combined with 10 (■) or 100 (▼) mg/kg of tilorone analogue 11,567 (BDD).

cyte populations (17, 23). Tilorone enhanced antibody production of the IgM and IgG classes in mice to both thymus-dependent (sheep red blood cells) and thymus-independent (*Escherichia coli* endotoxin) antigens (15). This compound also served as an adjuvant that significantly enhanced influenza hemagglutination-inhibition antibody titers in guinea pigs when injected together with, or at separate sites from, subunit influenza vaccine (14). In contrast, adjuvant studies with BDD and VEE vaccine (D. G. Harrington, unpublished observations) indicated that significant immunopotentiation was not obtained in mice when vaccine was inoculated sc and BDD was given ip or *per os*.

In this study, BDD was shown to immunopotentiate VEE vaccine in monkeys and mice. Monkeys given VEE vaccine containing 10 mg/kg of BDD developed higher primary (4-fold) and secondary (12-fold) geometric mean antibody titers than those administered vaccine with adjuvant. Mice given undiluted VEE vaccine containing BDD developed 3- to 4-fold greater antibody titers than vaccine controls. The immunopotentiation effect of BDD was most clearly demonstrated when it was given with a marginally antigenic vaccine dose. Graded doses of BDD (7 to 500 mg/kg) with a marginally antigenic vaccine dose of VEE vaccine (diluted 1:4) induced antibody production, whereas vaccine alone did not. Antibody titers were significantly higher than in vaccine controls when 125 ($P < 0.001$) or 250 ($P < 0.05$) mg/kg of BDD was given with the vaccine.

Survival of mice challenged 14 days after vaccination reflected the immunopotentiation effect of BDD. Mice administered undiluted VEE vaccine containing BDD had a significantly greater percentage survival ($P < 0.05$) than vaccine controls groups, although survival was not significantly greater than FCA or FIA vaccine groups. However, when BDD was used with marginally antigenic doses of vaccine, survival was significantly greater ($P < 0.05$) than when FCA or FIA were used as adjuvants.

The minimal dose of BDD which enhanced antibody response varied markedly in mice and monkeys. In mice, the minimal dose of BDD required to significantly potentiate antibody response (125 mg/kg) was near the safety limits, since skin lesions occurred when ≥250 mg/kg were injected sc. However, toxic deaths did not occur when 500 mg/kg was injected sc. Similarly, Kuehne *et al.* (7) determined that the effective antiviral dose of BDD by ip injection (250 mg/kg) was near the acute toxic dose level (500 mg/kg).

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