Delta-9-Tetrahydrocannabinol Decreases Host Resistance to Herpes Simplex Virus Type 2 Vaginal Infection in the B6C3F1 Mouse

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SUMMARY

The effect of delta-9-tetrahydrocannabinol (Delta-9-THC) on host resistance to herpes simplex virus type 2 (HSV-2) vaginal infection in the B6C3F1 mouse was determined. Animals were given Delta-9-THC or vehicle on days -1 to 2, or cyclophosphamide on day -1 or on days -1 to 2. HSV-2 was introduced intravaginally on day 0. Host resistance to virus infection was assessed by comparing frequency and severity of lesions, virus shedding and mortalities. Replicate groups of animals were bled on days 5, 8, 10, 14 and 21 post-viral inoculation to allow for screening for viraemia and for definition of the effect of Delta-9-THC on the humoral response. Animals were also employed for determination of delayed hypersensitivity responses (DHR). Virus-infected animals treated with 100 mg/kg Delta-9-THC exhibited greater severity of herpes genitalis, higher mortalities and higher mean titres of virus shed from the vagina. Suppression of the humoral response to HSV-2 occurred in animals treated with this dose of drug when compared to virus-infected vehicle controls. A delay in the onset of the DHR to HSV-2 was observed in animals receiving 100 mg/kg Delta-9-THC when compared with those receiving vehicle. These results indicate that Delta-9-THC decreases host resistance to HSV-2 vaginal infection in the B6C3F1 mouse. This decreased resistance is associated with suppression of the immune response to primary infection with HSV-2.

INTRODUCTION

Cannabis, or marijuana, has been shown to induce a variety of immunosuppressive and inhibitory activities both in vitro and in vivo. Marijuana elicits dysfunction in lymphocyte response to mitogens and particulate antigens (Nahas et al., 1974), decreases T-cell rosette formation (Gupta et al., 1974; Cushman & Khurana, 1976), suppresses leukocyte migration (Schwartzfarb et al., 1974), and affects alveolar macrophage morphology, function and motility (Mann et al., 1971). Delta-9-tetrahydrocannabinol (Delta-9-THC) is the major psychoactive component of marijuana. This drug inhibits cellular RNA, DNA and protein synthesis (Blevins & Regan, 1976; Pringle & Bradley, 1978). We have previously demonstrated that Delta-9-THC decreases host resistance to herpes simplex virus type 2 (HSV-2) vaginal infection in the guinea-pig (Cabral et al., 1985). In these studies, virus infected animals treated with the drug exhibited increased frequency and severity of genital lesions, shed higher titres of virus from the vagina and experienced greater mortalities. Although the guinea-pig remains the animal of choice as a model of human herpes genitalis, the more fully characterized immune system of the mouse renders the latter ideal for defining elements of host resistance which may be targeted by cannabinoids. Thus, the present investigation was undertaken to determine whether Delta-9-THC decreases host resistance to HSV-2 vaginal infection in the B6C3F1 mouse. Assessment of host resistance to HSV-2 vaginal infection in Delta-9-THC-treated and untreated mice was accomplished by comparing the incidence and severity of lesions, by quantification of virus shed
from the vagina and by tabulation of animal mortalities. The effect of Delta-9-THC on host immunity was determined by quantification of antiviral antibodies and by measurement of the delayed hypersensitivity response (DHR) to HSV-2.

METHODS

Animals. Female B6C3F1 mice were purchased from Charles River Breeding Laboratory (Wilmington, Mass., U.S.A.). Animals were quarantined for 1 week before initiation of experiments. Mice used in experiments were 8 to 9 weeks old. Animals employed in vaginal virus-shedding experiments were coded by ear-punching.

Drugs. Delta-9-THC was supplied by the National Institute of Drug Abuse, Rockville, Md., U.S.A. Delta-9-THC was employed in this study since it is the major psychoactive component of cannabis and induces immunosuppression. The drug was prepared as a solution in Emulphor (EL-360; GAF Corp., New York, N.Y., U.S.A.) and ethanol (1:1, w/v) at a stock concentration of 100 mg/ml. The Delta-9-THC was diluted with 0.85% NaCl to yield final concentrations of 20, 10, 5 and 1.5 mg/ml for mouse inocula. The vehicle control consisted of Emulphor/ethanol/saline (1:1:8). Drug and vehicle solutions were administered intraperitoneally such that each mouse received 0.01 ml/g body weight.

Cells. Green monkey kidney (Vero) and HEp-2 cells were grown in Eagle's MEM containing 10% Hanks' balanced salts (HMEM), 10% foetal calf serum and 0.2% antibiotic/antimycotic (Gibco).

Virus. HSV-2, strain P180/HSV-2/ST (FMC), was isolated from a gynaecological patient and was propagated in Vero cells. The virus was typed as HSV-2 on the basis of fingerprinting of viral DNA by partial EcoRI restriction endonuclease analysis (Hamelin et al., 1984). The propagated FMC strain of HSV-2 was shown by plaque assay (Rapp, 1963) to have a stock titre of $1 \times 10^9$ p.f.u./ml and was used for infectivity and mortality studies. Laboratory strain HSV-2 (186) was propagated in HEp-2 cells and contained $1 \times 10^8$ p.f.u./ml. HSV-2 strain 186 was used primarily for vaginal inoculation of mice employed in antibody and DHR studies.

Virus titrations. Virus was titrated by plaque assay on Vero cell monolayers grown in 24 well Costar cluster plates. Vaginal tissues of etherized mice were gently abraded with a scalpel prior to introduction of 0.05 ml HSV-2 containing $5 \times 10^4$ to $5 \times 10^7$ p.f.u./0.05 ml depending on the study under investigation. Animals employed in herpes genitalis and mortality studies received an inoculation of HSV-2 strain FMC containing $1 \times 10^5$ p.f.u./0.05 ml. Animals employed in antibody studies received an inoculum of HSV-2 strain 186 containing $1 \times 10^5$ p.f.u./0.05 ml. Animals utilized for DHR studies were inoculated with either $5 \times 10^4$ p.f.u./0.05 ml or $1 \times 10^5$ p.f.u./0.05 ml of HSV-2 strain 186 or with $1 \times 10^4$ p.f.u./0.05 ml of HSV-2 strain FMC. HSV-2 strain 186 was employed in the latter studies since this laboratory strain elicits disease which is characterized by less severity and lower mortality than that elicited by the wild-type FMC strain. The use of HSV-2 strain 186 enabled long-term studies to be carried out aimed at defining the effect of Delta-9-THC on the immune response while simultaneously minimizing animal mortalities as a result of wild-type virus infection. Following virus inoculation, the vaginal orifice was plugged with Gel-Foam (Upjohn, Kalamazoo, Mich., U.S.A.) to prevent leakage. The Gel-Foam has been shown to be inert and dissolves in 2 to 3 days.

Virus shedding. Vaginal secretions were collected by swabbing the vagina with cotton-tipped applicator sticks pre-moistened with HMEM. Vaginal swabs were placed in vials containing 1 ml HMEM supplemented with antibiotics (penicillin/streptomycin/fungizone) and vortexed. Vials were stored at $-80^\circ$C until assayed for virus. At the time of assay, swab suspensions were diluted in HMEM, passed through sterile 0.22 μm Acrodisc filters (Gelman) and inoculated onto Vero cell monolayers. Virus infectivity titres were determined by plaque assay as described above.

Antigen for delayed hypersensitivity studies. HSV-2 (FMC)-infected HEp-2 cells were harvested when cultures exhibited 90% c.p.e. and were subjected to three freeze–thaw cycles. The suspension was then diluted in HMEM and Dounce-homogenized using a loose-fitting pestle. The homogenate was sonicated at 7 kHz for 1 min and the sonicate was centrifuged at 2500 r.p.m. for 30 min (4°C) using a Beckman TJ-6 centrifuge. An aliquot of supernatant containing HSV-2 was removed for subsequent titration of infectivity. The remaining supernatant material was inactivated for 30 min using a u.v. light (UltraViolet Products, San Gabriel, Ca., U.S.A.) at a distance of 1 cm. Both non-inactivated and u.v.-inactivated virus were titrated by plaque assay. The antigen preparation contained $2 \times 10^8$ p.f.u./ml prior to inactivation. No plaques were elicited in Vero cell cultures inoculated with the u.v.-inactivated virus.

Host resistance studies. In preliminary studies, uninfected mice were given Delta-9-THC in doses ranging from 15 to 200 mg/kg in order to define a drug regimen free of measurable toxicological effects. Animals receiving 200 mg/kg of drug exhibited drug-induced morbidity which included hypothermia and weight loss. Thus, for host
resistance studies mice receiving Delta-9-THC were given 15, 50 or 100 mg/kg of drug. Animals given cyclophosphamide received 50 mg/kg. In each experiment, animal groups were included to serve as virus controls and received either HSV-2 or the drug vehicle plus HSV-2. All drugs and vehicle were administered intraperitoneally for 4 consecutive days beginning on the day prior to intravaginal administration of 1 x 10^5 p.f.u./0.05 ml HSV-2 (FMC strain). Animals were observed at the time of dosing for pharmacotoxicological signs. Mice were monitored for morbidly and mortality daily for 21 days following administration of virus. Vaginal swabs were obtained from all mice infected intravaginally on days 4, 7, 10, 14 and 21 post-viral inoculation in order to monitor virus shedding. Animals that died were necropsied, and lung, liver and brain tissues were recovered for subsequent quantification of HSV-2 by plaque assay. These tissues were stored in HMEM containing three times antibiotics at -80°C until assayed for virus.

Delayed hypersensitivity response (DHR). Mice were placed into groups to receive either 100 mg/kg Delta-9-THC, 15 mg/kg Delta-9-THC, vehicle or no treatment. Three experiments were performed. In an initial experiment, to determine the kinetics of response, HSV-2 strain 186 was administered intravaginally in a 0.05 ml vol. containing 5 x 10^6 p.f.u. A cell control group received 0.05 ml of HeP-2 cell homogenate processed identically to virus stock suspensions. In a second experiment mice were given 1 x 10^7 p.f.u./0.05 ml HSV-2 (FMC). Mice of the third experiment received 1 x 10^7 p.f.u./0.05 ml HSV-2 (FMC). In all three experiments drug administrations were performed on days -1 to 2 with virus or HeP-2 inocula introduced on day 0. In experiment 1, mice of each group were injected with 0.02 ml HSV-2 antigen (4 x 10^6 p.f.u. of u.v.-inactivated HSV-2) in the right footpad on days 6, 8, 10 and 15 post-inoculation. Animals of the second and third experiments received antigen on day 8 post-inoculation since this time period was shown to yield optimal DHR. All animals received 0.02 ml of control antigen (i.e. uninfected cell homogenate) in the left footpad. In all experiments, at 24 h following antigen challenge, mice were injected intraperitoneally with 2 x 10^6 c.p.m. of 125I-labelled human serum albumin (125I-HSA) (sp. act. 8.5 gCi/mg; Mallinckrodt, St. Louis, Mo., U.S.A.). Two h following 125I-HSA inoculation, mice were sacrificed by cervical dislocation and their left and right feet were amputated. Feet were counted for radioactivity using an LKB gamma counter (LKB). Sensitization ratios (SR) were calculated as c.p.m. of sensitized foot/c.p.m. of unsensitized foot. Positive responses were defined as those SRs exceeding the 99% confidence interval for antigen-sensitized, non-HSV-2-infected controls.

Serological studies. Mice were inoculated intraperitoneally with 15 or 100 mg/kg Delta-9-THC for 4 consecutive days beginning on the day prior to vaginal administration of virus. Control animals received drug vehicle or 200 mg/kg cyclophosphamide (only one inoculum given on the day prior to virus inoculation). One group of control animals received no intraperitoneal injection. Virus-infected animals were inoculated intravaginally with 0.05 ml HSV-2 strain 186 containing 1 x 10^5 p.f.u. and the vaginal vault was sealed with Gel-Foam. Individual groups of animals were bled by cardiac puncture on days 5, 8, 10, 14 and 21 post-viral inoculation. Sera were stored at -80°C until assayed for viraemia by plaque assay and for antiviral antibodies by neutralization assay. complement fixation (CF) and ELISA. Sera employed for antibody measurements were heat-inactivated at 56°C for 30 min.

Antibody titration by virus neutralization. A complement-dependent microneutralization assay to assess anti-HSV-2 neutralizing antibodies was performed as previously described (Morahan et al., 1981). Plates were scored following fixation in absolute methanol containing 5% (v/v) Wescodyne (West Chemical Products, Princeton, N.J., U.S.A.) and staining with 0.5% aqueous crystal violet containing 0.5% (v/v) Photo-Flo 200 (Eastman Kodak). The last dilution showing less than 10% c.p.e. was taken as the endpoint. Scoring of stained cultures was employed for calculation of neutralizing antibody titre to HSV-2.

Antibody titration by CF. A CF test employing a microtitre block titration as described by the Centers for Disease Control, Atlanta, Ga., U.S.A. was employed (P.H.S. publication no. 1228). Controls were included to monitor for antigen anti-complementary activity and for antibody anti-complementary activity. Additional controls consisted of complement back titrations and cell controls.

Antibody titration by ELISA. Sera were tested for IgM antibodies to HSV-2 by ELISA. Commercially prepared rabbit antiserum to mouse IgM conjugated to horseradish peroxidase (Cappel Laboratories, Cochranville, Pa., U.S.A.) was used. The solid-phase HSV and cell control antigens were linked to ferrous beads (Litton Bionetics, Charleston, S.C., U.S.A.) and were pretreated with normal goat serum diluted 1:100 in STB [0.05% saline pH 7.0, 0.05% Tween 20, 1% bovine serum albumin (BSA)].

Test sera were diluted 1:100 in STB and 0.2 ml aliquots were added to duplicate wells of a 96-well plate. HSV and control antigen-coated beads were then introduced to replicate wells using a magnetic transfer device (Litton Bionetics). Following a 90 min incubation at 37°C, the plates were washed 12 times in STB (4% BSA) to remove unreacted antibody. Horseradish peroxidase-conjugated goat anti-mouse IgM antiserum (0.2 ml) at a dilution of 1:200 was added to each well and the plates were incubated for 90 min. The plates were then washed 12 times with STB (4% BSA) and 0.2 ml of substrate containing 0.03% 2,2'-azoino-di-[3-ethylbenzthiazolesulphonate] (Litton Bionetics) and 0.004% H_2O_2 was added to each well. After 10 min at room temperature, the reaction was terminated by the addition of 0.025 ml 1:25% NaF. Plates were read for absorbance at 405 nm using an ELISA reader (Biotek Instruments, Burlington, Vt., U.S.A.). Each assay included a known anti-HSV-2-positive mouse
serum control and normal mouse serum controls. ELISA-specific activity was defined as absorbance (viral antigen)/absorbance (control antigen). Employing this formula, positive IgM responses were defined as those test sera demonstrating ELISA-specific activities exceeding the upper limits of the 99% confidence limit of normal serum controls.

Statistical analysis. Data on the frequencies of lesion expression and mortality were analysed using the Fisher Exact Test (Fisher, 1950). Bartlett’s test for homogeneity, parametric ANOVA, Dunnett’s multirange Test and Student’s t-test as described by Zar (1974) were employed for analysis of virus shedding and for sensitization indices. Dose-related times until death and virus shedding were analysed by linear regression analysis (Zar, 1974).

RESULTS

Pathogenesis of HSV-2 vaginal infection

Mice were inoculated intraperitoneally for 4 consecutive days with Delta-9-THC at doses of 15, 50 or 100 mg/kg to test the effect of the drug on the pathogenesis of HSV-2 vaginal infection. Control animals received vehicle or 50 mg/kg cyclophosphamide. Virus-infected animals received $1 \times 10^5$ p.f.u./0.05 ml HSV-2 (FMC strain) on day 2. Results of lesion expression, mortality and mean time to death are summarized in Table 1. Primary herpes genitalis was characterized by swelling and erythema followed by the appearance of vesicular lesions on the external genitalia of 25% of vehicle-treated animals. Lesions were noted on 30% of animals receiving either 15 or 50 mg/kg of drug. In contrast, 40% of animals receiving 100 mg/kg Delta-9-THC or cyclophosphamide expressed lesions. The expression of lesions occurred by day 8 post-intravaginal inoculation and was followed by ascending neuropathy in most animals as evidenced by hind-limb tremours and paralysis. Animals exhibiting ascending neuropathy subsequently died. No significant differences were noted in time to lesion expression following vaginal infection between virus-infected, drug-treated or vehicle controls.

Deaths were recorded for 15% of virus-infected animals receiving vehicle. In contrast, 30% of animals treated with 15 or 50 mg/kg Delta-9-THC, and 40% of animals treated with 100 mg/kg Delta-9-THC or with cyclophosphamide, died. With the exception of two vehicle-treated mice, all animals that expressed genital lesions succumbed to the HSV-2 vaginal infection. Calculation of mean time to death, however, indicated that significantly earlier times of death (8.25 ± 0.25 days post-infection) occurred among mice treated with 100 mg/kg Delta-9-THC (P < 0.05) when compared to vehicle controls (11.00 ± 0.58 days post-infection). Thus, a correlation between increasing Delta-9-THC administration and decreased host resistance, as monitored by cumulative mortalities ($r = 0.88$) and by mean time to death over a 21 day experimental period, was observed.

In order to monitor virus shedding, vaginal swabs were obtained from mice on days 4, 7, 10, 14 and 21 post-intravaginal virus inoculation. The frequency and titres of HSV-2 recovered from vaginal swabs are summarized in Table 2. Of the 5 days monitored, virus was shed only on days 4 and 7. Virus was isolated from vaginal swabs of 35% of vehicle controls, 60% of cyclophosphamide-treated mice, and 70 to 90% of mice treated with Delta-9-THC. Significantly higher frequencies of intravaginal virus shedding were recorded for mice treated with 50 mg/kg (90%; $P < 0.01$) and 100 mg/kg (80%; $P < 0.05$) Delta-9-THC when compared to vehicle-treated controls (35%). Cumulative HSV-2 shedding (i.e. on days 4 plus 7) increased in a dose-related fashion ($r = 0.90$). Significantly higher titres of HSV-2 were shed from animals treated with 100 mg/kg Delta-9-THC when compared to vehicle controls (Student’s t-test; $P < 0.05$). All animals expressing genital lesions were shown to shed intravaginal virus. A high degree of correlation between mortalities and HSV-2 vaginal titres ($r = 0.97$) was noted.

Animals that succumbed to infection were subjected to necropsy at time of death. Brain, liver and lung fragments were co-cultivated with Vero cell monolayers to allow for detection of resident virus. In all cases, HSV-2 was isolated from the brain, but not from the liver or lung.

Delayed hypersensitivity response

Three experiments were conducted to determine the effect of Delta-9-THC on the DHR to HSV-2 vaginal infection. Animals were given 100 or 15 mg/kg Delta-9-THC, or vehicle. The first experiment was conducted to allow for definition of the time to optimal response to virus
Cannabinoid decreases humoral and cellular immunity in the mouse

Table 1. Frequency of lesion expression, mortality and time to death (TTD) among mice treated with Delta-9-THC and exposed intravaginally to $1 \times 10^5$ p.f.u. HSV-2

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. of mice</th>
<th>% Lesion†</th>
<th>% Mortality†</th>
<th>TTD ± s.e. (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg/kg Delta-9-THC</td>
<td>10</td>
<td>40</td>
<td>40</td>
<td>8.25 ± 0.25</td>
</tr>
<tr>
<td>50 mg/kg Delta-9-THC</td>
<td>10</td>
<td>30</td>
<td>30</td>
<td>10.67 ± 0.88</td>
</tr>
<tr>
<td>15 mg/kg Delta-9-THC</td>
<td>10</td>
<td>25</td>
<td>25</td>
<td>9.33 ± 0.33</td>
</tr>
<tr>
<td>Vehicle§</td>
<td>20</td>
<td>25</td>
<td>15</td>
<td>11.00 ± 0.58</td>
</tr>
<tr>
<td>Cyclophosphamide§</td>
<td>5</td>
<td>40</td>
<td>40</td>
<td>11.50 ± 0.50</td>
</tr>
</tbody>
</table>

* Mice were acclimatized to the animal room for 7 days prior to administration of Delta-9-THC. The Delta-9-THC, vehicle or cyclophosphamide was administered intraperitoneally by four injections on consecutive days. Intravaginal infection of $1 \times 10^5$ p.f.u./0.05 ml HSV-2 was performed 1 day after the first injection of Delta-9-THC.
† Percentages of lesion expression or mortality observed within the 21 day experimental period.
§ Emulphor: ethanol (1:1).
¶ Mice received 50 mg/kg cyclophosphamide in 0.15 M-saline.
|| P < 0.05.

Table 2. Frequency and titre of HSV-2 recovered from vaginal swabs of mice treated with Delta-9-THC and exposed intravaginally to $1 \times 10^5$ p.f.u. HSV-2

<table>
<thead>
<tr>
<th>Treatment*</th>
<th>No. of mice</th>
<th>% Positive†</th>
<th>HSV-2 titre on‡</th>
<th>Cumulative titre§</th>
</tr>
</thead>
<tbody>
<tr>
<td>100 mg/kg Delta-9-THC</td>
<td>10</td>
<td>80†</td>
<td>Day 4</td>
<td>Day 7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3240 ± 1781-6</td>
<td>4570 ± 2253-4</td>
</tr>
<tr>
<td>50 mg/kg Delta-9-THC</td>
<td>10</td>
<td>90¶</td>
<td>1970 ± 1401-2</td>
<td>4900 ± 2030-4</td>
</tr>
<tr>
<td>15 mg/kg Delta-9-THC</td>
<td>10</td>
<td>70†</td>
<td>2710 ± 1514-1</td>
<td>2990 ± 1282-8</td>
</tr>
<tr>
<td>Vehicle§</td>
<td>20</td>
<td>35†</td>
<td>1155 ± 544-3</td>
<td>2195 ± 1043-9</td>
</tr>
<tr>
<td>Cyclophosphamide§</td>
<td>5</td>
<td>60†</td>
<td>80 ± 80-0</td>
<td>4400 ± 3206-9</td>
</tr>
</tbody>
</table>

* Mice were acclimatized to the animal room for 7 days prior to administration of Delta-9-THC. The Delta-9-THC, vehicle or cyclophosphamide was administered intraaperitoneally by four injections on consecutive days. Intravaginal infection of $1 \times 10^5$ p.f.u./0.05 ml HSV-2 was performed 1 day after the first injection of Delta-9-THC.
† Percentages of animals observed to shed virus.
¶ Vaginal swabs were obtained from all surviving mice on days 4, 7, 10, 14 and 21 post-infection. Swabs were inoculated (1:10 dilution) onto Vero cell monolayers. Titres are presented as p.f.u./ml ± s.e. No HSV-2 was detected on days 10, 14 or 21.
§ Cumulative virus shed was observed by linear regression analysis to increase in a linear manner ($r = 0.90$) over the dose range observed. Total virus shed was correlated with cumulative mortalities ($r = 0.97$).
|| P < 0.05.
¶ P < 0.01.

antigen challenge following HSV-2 intravaginal inoculation. Results of the frequency of positive response to HSV-2 antigen challenge (i.e. sensitization ratio > 1.17), as measured in mice from 6 to 15 days following inoculation with HSV-2 strain 186, are presented in Fig. 1. On day 6 post-viral inoculation, 16.7% (2/12) of vehicle-treated mice were found to respond to HSV-2 antigen challenge. The percentage of animals responding increased to 50% for days 8 (6/12) and 10 (3/6) post-viral inoculation. By day 15, the percentage of vehicle-treated animals eliciting a positive response decreased to 33.3% (2/6). Thus, day 8 was found to be the earliest time post-viral inoculation for which the maximum number of vehicle-treated animals responded to HSV-2 antigen challenge. Treatment of mice with 15 mg/kg Delta-9-THC resulted in a 42.9% (3/7) positive response by day 8 with a decline in the frequency of response to 28.6% (2/7) on day 10. In contrast, the frequency of positive response among animals administered 100 mg/kg Delta-9-THC declined steadily from days 6 to 10 post-viral inoculation. On day 8 only an 18.1% (2/11) positive response was recorded. On day 10, none of the animals treated with 100 mg/kg Delta-9-THC (0/5) was observed to respond to HSV-2 antigen challenge. However, by day 15, a positive response was observed in 40% (2/5) of the animals given 100 mg/kg Delta-9-THC.
Fig. 1. Effects of Delta-9-THC administration on the frequency of positive delayed hypersensitivity response to HSV-2. Animals were injected intraperitoneally with 100 (□) or 15 (○) mg/kg Delta-9-THC or vehicle (△) on days -1 to 2. Mice were exposed intravaginally to $5 \times 10^6$ p.f.u. of HSV-2 (strain 186) on day 0 and were challenged with the equivalent of $4 \times 10^6$ p.f.u. of u.v.-inactivated HSV-2 (strain FMC) in the right rear footpad on days 6, 8, 10 and 15. Mice received identical inocula of control antigen (uninfected cell homogenate) in the left footpad. Twenty-four h after antigen challenge, mice were given $2 \times 10^6$ c.p.m. of $^{125}$I-HSA intraperitoneally. Two h after $^{125}$I-HSA administration mice were sacrificed, their feet amputated and counted on a gamma counter. Positive responses were defined as those ratios of c.p.m. sensitized/c.p.m. control feet exceeding the 99% confidence interval for 14 antigen-sensitized uninfected controls (mean = 1.07, $0.972 < U < 1.167$). Animals receiving 100 mg/kg Delta-9-THC experienced a delay in DHR to HSV-2 when compared to virus-infected vehicle controls.

Fig. 2. Composite results of the effect of Delta-9-THC administration on the magnitude of the delayed hypersensitivity response. Mice were given vehicle (VH; 18 mice), 15 mg/kg Delta-9-THC (17 mice) or 100 mg/kg Delta-9-THC (21 mice) on days -1 to 2 and were exposed intravaginally to HSV-2 on day 0. All animals were challenged with u.v.-inactivated HSV-2 and control antigen on day 8, shown to be the time of optimal DHR response. A significantly lower mean response ($P < 0.05$) was recorded for mice treated with 100 mg/kg Delta-9-THC when compared to vehicle-treated HSV-2-infected animals.

In order to determine whether Delta-9-THC affected the DHR differentially with respect to the strain of HSV-2 employed, a second experiment was conducted in which animals were inoculated intravaginally with the wild-type HSV-2 strain, FMC, in addition to the laboratory strain, 186. No differences in the frequency, magnitude or time sequence of response in DHR were discerned with respect to virus strain employed for vaginal infection (data not shown). Composite results of experiments 1 and 2 which indicate the magnitude of response of HSV-2-infected animals given Delta-9-THC or vehicle, and which were tested for DHR on day 8 post-viral inoculation, are summarized in Fig. 2. Of 18 vehicle-treated animals, the mean SR was $1.42 \pm 0.16$. A mean SR of $1.22 \pm 0.06$ was observed for 17 animals treated with 15 mg/kg Delta-9-THC. In contrast, administration of 100 mg/kg Delta-9-THC resulted in a mean SR of $1.15 \pm 0.07$ in 21 mice tested. Thus, a significant reduction in the magnitude of DHR was observed in animals treated with 100 mg/kg Delta-9-THC (Student's $t$-test, $P < 0.05$) when compared to vehicle-treated, HSV-2-infected animals.

Serological studies

Animals were inoculated with 15 or 100 mg/kg Delta-9-THC for 4 consecutive days. Control animals received vehicle for 4 consecutive days or 200 mg/kg cyclophosphamide for 1 day. Animals were inoculated intravaginally with $0.05 \text{ ml HSV-2 strain } 186 \text{ containing } 1 \times 10^6 \text{ p.f.u. on day 2. Individual groups of animals were bled by cardiac puncture on days 5, 8, 10, 14 and 21 post-viral inoculation. Sera were tested for the presence of virus in the circulation by plaque assay. Virus was not found in the sera of any of the animals. Sera were then employed for measurement of humoral response to HSV-2 by virus neutralization, CF and ELISA.}
Cannabinoid decreases humoral and cellular immunity in the mouse

Fig. 3. Mean neutralization titres for HSV-2-infected mice treated with Delta-9-THC, vehicle or cyclophosphamide. Mice were given vehicle (■), 15 mg/kg Delta-9-THC (■■) or 100 mg/kg Delta-9-THC (□) on days −1 to 2 and were exposed intravaginally to 1 × 10⁵ p.f.u. HSV-2 (strain 186) on day 0. Animals receiving cyclophosphamide were injected with one dose (200 mg/kg) on day −1 (△). Representative groups of mice were bled by cardiac puncture on days 5, 8, 10, 14 and 21 and sera were heat-inactivated. Sera were employed in a complement-dependent microneutralization assay. By day 14 post-infection significantly lower titres (P < 0.05) of neutralizing antibody (NT) were observed in mice treated with 100 mg/kg Delta-9-THC when compared to virus-infected vehicle controls. By day 21 these lower titres in animals given 100 mg/kg Delta-9-THC were comparable to those elicited in virus-infected animals treated with cyclophosphamide (P < 0.01).

Antibody titration by neutralization

Results of mean neutralizing antibody titres elicited in virus-infected animals treated with Delta-9-THC, vehicle or cyclophosphamide are summarized in Fig. 3. Sera of vehicle-treated mice infected with HSV-2 contained relatively low titres of neutralizing antibody (i.e. less than 1:10) up to day 10 post-viral inoculation. From day 10 to day 21, however, a linear increase in mean antibody titre was recorded (r = 0.889), with greater than fourfold levels, relative to day 10, attained on day 21. A similar pattern of neutralizing antibody response was noted for virus-infected animals treated with 15 mg/kg Delta-9-THC. In contrast, virus-infected animals treated with 100 mg/kg Delta-9-THC did not exhibit significant neutralizing antibody titres to HSV-2 (i.e. greater than 1:10) at any time during the 21 day study period. Animals treated with cyclophosphamide exhibited a slight mean increase in antibody titre (i.e. 1:20) by day 10 post-viral inoculation. Antibody titres in these animals were found to decrease after that time, however, reaching less than 1:10 by day 21.

Antibody titration by CF

Results of mean CF antibody responses elicited in virus-infected animals treated with Delta-9-THC, vehicle or cyclophosphamide are summarized in Fig. 4. Sera of vehicle-treated mice infected with HSV-2 contained relatively high titres of CF antibody (i.e. mean = 128 ± 59.9) on day 5 post-viral inoculation. Lower mean titres were observed in vehicle-treated animals on day 8. Titres were then seen to increase in a linear manner from day 8 to day 14. By day 14, titres were seen to plateau at a mean of 232.5 ± 44.7. In contrast, animals treated with 100 mg/kg Delta-9-THC demonstrated a maximum mean titre of CF antibody on day 10 of 90-0 ± 38.4. This decrease was particularly evident on day 14 where significantly depressed CF titres (P < 0.01)
Fig. 4. Mean complement-fixing antibody titres for virus-infected mice treated with Delta-9-THC, vehicle or cyclophosphamide. Mice were given vehicle ( ), 15 mg/kg Delta-9-THC ( ) or 100 mg/kg Delta-9-THC ( ) on days −1 to 2 and exposed intravaginally to $1 \times 10^5$ p.f.u. of HSV-2 (strain 186) on day 0. Animals treated with cyclophosphamide received 200 mg/kg on day −1 ( ). Representative groups of mice were bled by cardiac puncture on days 5, 8, 10, 14 and 21 and sera were heat-inactivated. Sera were employed in a CF antibody assay. By day 14 post-infection significantly lower titres ($P < 0.01$) of CF antibody were observed in mice treated with 100 mg/kg Delta-9-THC and or with cyclophosphamide. By day 21 significantly lower titres in animals given 100 or 15 mg/kg Delta-9-THC, or cyclophosphamide ($P < 0.05$) occurred.

were observed when compared to vehicle controls. Animals treated with 15 mg/kg Delta-9-THC or with cyclophosphamide exhibited increasing CF titres beginning on day 8 post-viral inoculation. However, after that time, titres were seen to decrease. Antibody titres of mice treated with the lower dose of Delta-9-THC, or with cyclophosphamide, were either equal to, or were significantly lower than, those of virus-infected vehicle controls.

IgM antibody determination by ELISA

Mice were screened for IgM antibodies to HSV-2 by ELISA. A positive antibody response was defined as that ELISA reading exceeding the upper limit of the 99% confidence interval of readings of drug-treated uninfected (U) controls ($0.57 < U < 1.40$). No significant differences in the frequency of IgM responses were noted for any of the treatment groups. A maximum frequency of response occurred on day 8 post-infection for vehicle-treated animals where 25% (2/8) of the mice elicited anti-HSV-2 IgM antibodies. Animals treated with 15 or 100 mg/kg Delta-9-THC demonstrated maximum frequencies of IgM response on day 14 post-infection where 28.6% (2/7) of animals at each level of drug administration responded. A similar maximum response for IgM at this later time period occurred in animals treated with 200 mg/kg cyclophosphamide where a 25% (1/4 animals) response was noted.

DISCUSSION

The results of this study demonstrate that Delta-9-THC decreases host resistance to HSV-2 vaginal infection in the mouse. This decreased resistance was manifested by a dose-related trend towards increased cumulative mortalities and acceleration of the disease process in Delta-9-THC-treated mice. Doses as low as 15 mg/kg resulted in more severe herpes genitalis as evidenced by the greater quantities of virus shed from the vagina in infected animals. However, maximal decreased resistance was elicited at drug doses of 100 mg/kg. At this higher dose, mice experienced higher mortalities, expressed higher frequency of genital lesions and shed greater
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amounts of virus from the vagina when compared to virus-infected vehicle controls. This decreased resistance was expressed in the absence of measurable toxicological effects due to Delta-9-THC administration. Drug-induced hypothermia and weight loss, which resulted at higher dose administration (i.e. 200 mg/kg or higher), were not observed in the Delta-9-THC-treated, virus-infected animals. Furthermore, mortalities did not occur among non-virus-infected sentinel mice treated with 100 mg/kg Delta-9-THC. Quantification of virus shed from the vagina revealed a drug dose-dependent increase in the mean virus titre with a significant elevation of titre recorded for animals treated with 100 mg/kg Delta-9-THC when compared to virus-infected vehicle controls. These higher virus titres were recorded as early as 4 days post-vaginal virus inoculation. In contrast, minimum quantities of virus were obtained at this time from virus-infected animals that received cyclophosphamide, indicating that Delta-9-THC and cyclophosphamide affected host resistance differentially at this early time period. The Delta-9-THC, for example, may have enhanced virus replication at the primary site of infection. Indeed, experiments in vitro conducted in this laboratory have shown that Delta-9-THC elicits three- to fourfold greater quantities of virus in cultures treated with drug. Thus, increased virus replication may have contributed to the higher mortalities in mice receiving Delta-9-THC as a result of expression of greater numbers of localized lesions. These virus-containing lesions would facilitate destruction of epithelial cells, exposure of axon endings, infection of myelinated and unmyelinated fibres, and would enhance virus migration to nerve ganglia and the brain (Becker et al., 1984). The presence of virus in the brain of animals which succumbed to primary vaginal infection, concomitant with its absence from the blood, liver or lung, supports the hypothesis that the central nervous system was targeted by the virus. Comparable vaginal virus titres, however, were obtained from cyclophosphamide-treated and Delta-9-THC-treated animals on day 7. Furthermore, equal frequencies of lesion expression and mortality were recorded for cyclophosphamide-treated animals and animals receiving 100 mg/kg Delta-9-THC. These results suggest that Delta-9-THC may also contribute to the decreased resistance in the virus-infected mice by inducing immunosuppression.

To test whether Delta-9-THC targeted the immune system in HSV-2-infected mice, both cellular and humoral immunity were examined. Since cell-mediated immunity has been ascribed a major role in host resistance to primary vaginal HSV-2 infection (Morahan et al., 1977), the effect of Delta-9-THC on DHR was investigated. In mice treated with 100 mg/kg Delta-9-THC, the DHR was delayed or inhibited through the period of primary acute infection (i.e. days 8 and 10) and then rose, by day 15, to levels comparable to those of vehicle controls which had been attained as early as 8 days post-infection. Additionally, measurement of DHR, in three replicate experiments on day 8, demonstrated a significantly lower response in mice treated with 100 mg/kg Delta-9-THC when compared to vehicle controls. This lower response to cellular immunity to HSV-2 at this early time period (i.e. 8 days post-infection) corresponded to the mean time to death in animals receiving 100 mg/kg Delta-9-THC. These results suggest that drug administration resulted in a reduction of T lymphocyte-dependent cell-mediated immunity, an effect previously found to be associated with Delta-9-THC administration (Klykken et al., 1977; Smith et al., 1978). Whether the drug affects the afferent or efferent branch of cell-mediated immunity remains to be determined. Nevertheless, the delay in DHR response during the primary infection process, accompanied by enhanced virus replication, would compromise host resistance to the HSV-2 infection.

Administration of Delta-9-THC resulted in significant reductions in antibody titres to HSV-2 infection. Animals infected with HSV-2 and treated with vehicle expressed fourfold increases in virus-neutralizing antibody titres between days 10 and 21 post-infection. In contrast, minimal neutralizing antibody titres were elicited between days 10 and 21 in animals receiving 100 mg/kg Delta-9-THC. These lower levels in the cannabinoid-treated animals were comparable to those found in animals treated with cyclophosphamide. CF antibody responses to HSV-2 generally paralleled those of neutralizing antibodies in infected animals. Significantly lower CF antibody titres were recorded between days 14 and 21 in mice treated with 100 mg/kg Delta-9-THC when compared to virus-infected vehicle controls. HSV-2 neutralizing and CF antibodies have been observed in several animal models of virus infection (Morahan et al., 1979; Munson & Fehr,
While these antibodies have not been found to preclude de novo infection with HSV-2, or to prevent recurrence of latent infection, they have been shown to limit dissemination of virus within the host and to modify the severity and duration of recurrent infection (McKendall et al., 1979). Thus, Delta-9-THC administration may limit host resistance to reinfection with HSV-2. Studies are presently in progress to define the effect of this cannabinoid on challenge infection with HSV-2 in animals previously exposed to the virus.

In the present study, transient elevations in CF and neutralizing antibody titres were observed by day 5 in vehicle-treated animals, suggestive of anti-HSV-2 IgM activity. Antibody titres then declined and did not reach appreciable levels until day 10 post-infection, after the time of peak morbidity and mortality of primary acute infection had occurred. In order to determine the effect of Delta-9-THC on anti-HSV-2 IgM production, an ELISA employing anti-μ chain-specific anti-mouse IgG was employed. Vehicle control animals elicited IgM antibodies to HSV-2 as early as day 8 post-vaginal inoculation. In contrast, IgM responses to HSV-2 were not recorded until day 14 post-inoculation for animals treated with Delta-9-THC. These results suggest that cannabinoid administration delayed anti-HSV-2 IgM response to HSV-2. However, these data require further substantiation since the frequency of animals shown to elicit an IgM response to HSV-2 was relatively low. The relatively low frequency of IgM responses may have been a natural consequence of ‘localized’ HSV-2 vaginal infection.

The role of Delta-9-THC in suppression of other parameters of early host resistance such as interferon, natural killer cell activity and intrinsic macrophage activity is currently under investigation.

These results suggest that Delta-9-THC exerts a twofold effect on HSV-2 genital infection. First, the drug enhances virus replication. Second, the drug delays cell-mediated immunity and antibody response to the virus during primary infection. The consequences of these two effects are greater severity of herpes genitalis and facilitated access of virus to the brain leading to higher mortalities.

The doses of cannabinoid which effected a decreased host resistance to HSV-2 vaginal infection in the B6C3F1 mouse ranged from 15 mg/kg to 100 mg/kg. The mouse is less susceptible to Delta-9-THC than man. A part of this difference can be accounted for by the basis for expressing drug dose (mg/kg versus mg/m²). A dose of 100 mg/kg in the mouse corresponds to a dose of 10-2 mg/kg in man assuming equivalency on the basis of mg/m² (Klaassen & Doull, 1980). Delta-9-THC intake of as much as 15 mg/kg/day has been observed in some human users (Tennant & Groesbeck, 1972). Thus, the doses of cannabinoid that induced decreased resistance to HSV-2 in the mouse are comparable to those attainable in man.

Although the final relationship between genital HSV-2 infection in humans and marijuana use will require epidemiological investigation, studies employing the mouse vaginal model will help to identify the site of action of Delta-9-THC, and will permit definition of the mechanism by which this drug decreases host resistance to HSV-2 genital infection.

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