The Adverse Physiological Effects of Marihuana Use

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JOHN FRANCIS PAPARIELLA
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St. Meinrad Seminary
College of Liberal Arts
St. Meinrad, Indiana

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INTRODUCTION

The increasing prevalence of marihuana use in this country necessitates a serious investigation into the physiological effects of this practice. This study is a collection of primary research papers on the physiological effects of marihuana use in man. An annotated bibliography provides a comprehensive index, and a closing statement on the current status of marihuana use is offered. Marihuana research is in its very early stages. Tetrahydrocannabinol (THC) is the principal psychoactive ingredient in marihuana, and this discovery was made in 1965 by Mechoulam. The problems in compiling this presentation are largely due to the paucity of available data on the direct effects of marihuana use on man. Careful definition of dosage levels (short-term vs. chronic use) is uncertain. The abundance of conflicting research data adds to the confusion. There is intensive current research on marihauna use, and this study can only be a presentation of what is known at this time.

This study is a carefully limited selection of research papers. Mechoulam's (1973) book is an excellent resource for a serious study of the chemistry, pharma-

cology, metabolism, and clinical effects of marihuana use. The importance of the physiological and behavioural effects of marihuana use has generated several excellent reviews (Bonnie and Whitebread, 1974; Grinspoon, 1969; Powell, 1971; and Schultes, 1969). As a guide to the current literature on marihuana, the reader may wish to examine some of the bibliographies (Gamage et al., 1969; Liberman, 1971; Moore, 1969; and Waller et al., 1976).

1. Dose-Response Relationships to Cannabis in Human Subjects (Kiplinger and Manno, 1971).

Dose-response relationships of marihuana smoking and physiological effects were evaluated on male volunteers between the ages of 21 and 30. The volunteers smoked marihuana cigarettes which were pre-measured at varying doses. Pulse rate, subjective effects, motor performance, and verbal performance were measured. Direct dose-response relationships were recorded for pulse rate and subjective effects. Tachycardia, a very rapid and volatile contraction of the heart, was the most consistent response to marihuana use.

2. Effects of Marihuana Use on Body Weight and Caloric Intake in Humans (Greenberg, et al. 1976)

Body Weight and caloric intake were measured in a group of heavy and casual marihuana users before and after 21 days of marihuana smoking. Both groups had significant increases in caloric intake and weight gain during the testing period. Water retention, as measured by urine output, was not the reason for weight gain.

3. Inhibition of Glucose Efflux from Human Erythrocytes by Hashish Components (Schurr, et al. 1974).

"Marihuana hunger" (craving for sweets) is not caused by a lowered blood glucose level. Glucose transport across cellular membranes was tested on freshly drawn human blood. Glucose efflux from human erythrocytes is inhibited by tetrahydrocannabinol (THC). This inhibition results in a temporary halt in glucose transport from plasma into the erythrocytes. The adverse interaction of hashish components and cellular membranes of erythrocytes is the reason for the inhibition of glucose efflux from human erythrocytes.

4. Delta-8- and Delta-9- Tetrahydrocannabinol: Effects on Cultured Human Leucocytes (Neu, et al. 1974).

Tetrahydrocannabinol (THC) was added to human blood cultures. The presence of THC resulted in a drastic decrease in the mitotic index at all concentrations.

Less than 5% of the metaphases in the cultures exposed to THC had gaps and breaks. THC did not cause visible damage to human lymphocyte chromosomes in vitro. Nothing was determined concerning possible in vivo effects of the drug.

5. The Effect of Delta-9- Tetrahydrocannabinol on the Chromosomes of Human Lymphocytes in vitro (Stenchever and Allen, 1972).

Clinical observations indicate a higher number of chromosome breaks for marihuana users than nonusers. Human leukocytes from 4 healthy donors were exposed to THC in tissue cultures. There was no increase in the incidence of chromosome breaks or gaps in any of the study cultures. The in vitro results are not correlated with possible in vivo effects.

6. The Inhibition of DNA Synthesis by Cannabinoids (Carchman, Harris, and Munson, 1976).

In vitro tests on lung tumors grown in mice, and bone marrow tests on mice prove the inhibition of DNA synthesis by cannabinoids. The in vitro results with the cannabinoids were supported by in vivo tumor inhibition studies. THC was the only cannabinoid that inhibited both DNA synthesis in vitro and tumor growth in vivo.

7. Marihuana: Studies on the Disposition and Metabolism of Delta-9- tetrahydrocannabinol in Man (Lemberger, et al. 1970).

THC was administered intravenously to human volunteers. The drug persisted in plasma for several days. The metabolites of THC were excreted via the urine and feces for more than 8 days. THC persists in plasma for long periods. The drug is a non-polar compound, and it has a high affinity for fat tissue and lung tissue.

8. The Physiologic Disposition of Marihuana in Man (Lemberger and Rubin, 1975).

THC was administered orally to human volunteers. THC is lipid soluble; it is stored in fat tissue and lung tissue. THC also binds to tissue protein. THC is rapidly converted to a hydroxyl compound, and this compound has a more potent psychopharmacologic effect than the parent compound. More research is needed on the various metabolic products of THC.

9. Biological Disposition of tetrahydrocannabinoids (Truitt, 1971).

When intravenously administered, radiolabeled THC accumulates in the liver. THC and its metabolites are excreted via the urine and feces. The drug and its metabolites easily cross the placenta. There is a lack of preference of the drug for brain tissue. THC is quickly converted to a hydroxyl compound, and this compound exceeds the precursor in potency.

10. Delta-9-Tetrahydrocannabinol: Localization in Body Fat (Krem and Axelrod, 2973).

THC was injected subcutaneously in rats daily for 1 to 26 days. There was ten times as much of the drug in fat tissue as in other tissue. The drug also accumulated in brain tissue. The metabolite, 8,11-dihydroxy THC, has a much higher affinity for liver tissue than lung tissue. After a single injection, the drug persisted in fat tissue for 2 weeks.

11. Toxicity Testing <u>in vitro</u>. I. The Effects of Delta-9-tetrahydrocannabinol and Aflatoxin B, on the Growth of Cultured Human Fibroblasts (Cooper and Goldstein, 1976).

THC was added to strains of cultured human fibroblasts to test the toxicity of the drug. The lowest dose of THC producing a toxic response in the culture system is a much higher level than the level of THC at which humans experience the psychoactive effect. THC accumulates in fat and brain tissue; the toxicity of the drug at high levels is a potential danger.

12. Inhibition of Cellular Mediated Immunity in Marihuana Smokers (Nahas, et al. 1974).

Nahas tested the lymphocyte response in 51 young chronic marihuana smokers. Their lymphocyte response to foreign elements was significantly decreased and comparable to that of patients in whom impairment of T (thymus-derived) cell immunity is known to occur. The marihuana smokers had a higher rate of chromosome breakage.

13. Inhibition of a Lymphocyte Membrane Enzyme by Delta-9-tetrahydrocannabinol *in vitro* (Greenberg, et al. 1976).

In vitro tests were conducted with lysolecithin acyl transferase, which is a membrane-bound lymphocyte structure, and its level in T (thymus-dependent) lymphocytes is quickly increased by mitogens. The presence of mitogens causes an increase in phospholipid fatty acid turnover. This increase is the result of acyl transferase activity. THC inhibits this enzyme activity. THC causes changes in the lipid phase of the lymphocyte membrane. These changes inhibit the membrane-bound enzyme.

14. Mitogen-Induced Blastogenic Responses of Lymphocytes from Marihuana Smokers (White, Brin, and Janicki, 1975).

In vitro blastogenic responses to foreign elements were tested in microcultures of peripheral blood lymphocytes from chronic marihuana smokers. The functional response of the blood lymphocytes was not altered by long-term smoking of marihuana. There was no deleterious effect on the T (thymus-dependent) and B (thymus-independent) lymphocytes.

15. Normal Skin Test Responses in Chronic Marihuana Users (Silverstein and Lessin, 1974).

Immunocompetence was tested in 22 chronic marihuana smokers. *In vivo* skin testing revealed that marihuana smoking has no adverse effect on cell-mediated

immunity. Skin testing closely correlates to clinical prognosis in cancer patients. Skin testing is a reliable (96% successful) method of testing for immunocompetence.

16. Intact Humoral and Cell-Mediated Immunity in Chronic Marijuana Smoking (Rachelefsky, et al. 1971).

Twelve chronic marihuana smokers smoked marihuana daily for 64 consecutive days. The lymphocyte response to foreign agents was normal for B (thymus-independent) and T (thymus-dependent) cells. Chronic marihuana smoking has no adverse effect on cell-mediated immunity.

17. Impairment of Rosette-Forming T Lymphocytes in Chronic Marihuana Smokers (Grieco and Cushman, 1974).

Lymphocytes were obtained from 23 chronic marihuana smokers. T cells (thymus-dependent) were functionally tested on their ability to form rosettes (bind to sheep red blood cells). This is a test to determine the functional response of T lymphocytes which are responsible for cell-mediated immunity. The T lymphocytes from the chronic marihuana smokers had impaired rosette-forming ability. This test indicates that chronic marihuana smoking suppresses the T lymphocyte subpopulation.

18. Phytohemagglutinin-Induced Lymphocyte Transformation in Humans Receiving Delta-9-Tetrahydrocannabinol (Lau, et al, 1976).

Eight chronic marihuana smokers (ages 21-30) volunteered to ingest pre-measured amounts of THC. Blood samples were periodically drawn for lymphocyte response to the foreign substance-phytohemagglutinin.

19. Subacute Effects of Heavy Marihuana Smoking on Pulmonary Function in Healthy Men (Tashkin, et al. 1976).

Pulmonary tests were conducted on 28 experienced marihuana smokers. The subjects smoked daily for 47 to

59 days. After this period, several breathing tests revealed a significant decrease in pulmonary function. Prolonged heavy smoking of marihuana has an adverse effect on pulmonary function in human subjects.

20. Effects of Marijuana and Tobacco Smoke on DNA and Chromosomal Complement in Human Lung Explants (Leuchtenberger, Leuchtenberger, and Ritter, 1973).

Lung explants were exposed to the smoke of marihuana cigarettes. The tested explants had cells with deviating DNA content and chromosomal numbers. These results were observed shortly after exposure, and the changes persisted for a long time.

21. Effects of Marijuana and Tobacco Smoke on Human Lung Physiology (Leuchtenberger, Leuchtenberger, and Schneider, 1973).

Human lung explants were exposed to the smoke from marihuana cigarettes. The cells of the exposed lung explants had a decrease of mitosis and DNA synthesis. Cell death occurred in many of the exposed cells.

22. Marihuana Smoking: Cardiovascular Effects in Man and Possible Mechanisms (Beaconsfield, Ginsburg, and Rainsbury, 1972).

Eighteen doctors, who had never smoked marihuana, volunteered for the study. Tachycardia and increased limb blood flow occurred shortly after inhalation of marihuana. Marihuana smoking impairs vascular reflex responses.

23. Cardiovascular Effects of Prolonged Delta-9-tetrahydrocannabinol Ingestion (Benowitz and Jones, 1975).

Twelve male subjects volunteered to undergo cardiovascular tests following prolonged THC ingestion. The subjects had a decrease in heart rate and blood pressure; these effects were reversible. Circulatory responses to standing and exercise were impaired. All of the men gained weight because of fluid retention.

24. Cannabinoids Inhibit Testosterone Secretion by Mouse Testes in vitro (Dalterio and Burstein, 1977).

Decapsulated mouse testes were exposed to THC and cannabinol. The presence of THC inhibited the accumulation of testosterone in the culture medium. Cannabinol inhibited the production of testosterone. THC did reduce testosterone biosynthesis in the in vitro tests. Subcutaneous injections of cannabinol in mice reduced the plasma level of testosterone.

25. Delta-9-Tetrahyhydrocannabinol: Effects on Mammalian Nonmyelinated Nerve Fibers (Byck and Ritchie, 1973).

In vitro tests were conducted on a rabbit's vagus nerve tissue which was exposed to THC. The compound action potential of the nonmyelinated fibers was reduced. There was a very small slowing in conduction velocity. There was no adverse effect on the sodium pump.

MARIHUANA SMOKING

Cardiovascular Effects in Man and Possible Mechanisms

PETER BEACONSFIELD, M.D., PH.D., JEAN GINSBURG, M.A., D.M., AND REBECCA RAINSBURY, M.D.

Abstract Marihuana smoking by subjects without previous experience causes an increase in limb blood flow concomitantly with a rise in pulse rate. These responses are still evoked after administration of atropine but not after pretreatment with propranolol, a beta-adrenergic blocker. The tachycardias of atropine and of epinephrine are potentiated by marihuana. These findings suggest that the increase in pulse rate and peripheral blood flow induced by cannabis involves beta-adrenergic vascular mechanisms, and counsel caution in the administration of vasoactive drugs and anesthetics for those who may have been smoking marihuana.

THE only invariable objective measurable change hitherto recorded after marihuana smoking in man is tachycardia; there are few data concerning other cardiovascular responses to cannabis and, in particular, no information about its influence on peripheral blood flow. This lack of information is all the more surprising in that the tachycardia has been variously ascribed to release of epinephrine and altered autonomic activity,[1] to central excitation[2] and depression,[3] and to an atropine-like effect[4] — all this in the absence of laboratory studies either in animals or in man.

An opportunity fortuitously presented itself during a recent visit to Asia to study some effects of marihuana smoking on fundamental physiologic and biochemical responses in man. Our interest was in cardiovascular responses to marihuana smoking in man, with particular reference to changes in limb blood flow, and the possible role of adrenergic mechanisms and of efferent vagal activity in determining the observed effects.

MATERIAL AND METHODS

The subjects were healthy volunteer doctors, nine men and one woman, between 30 and 40 years of age, who were members of the staff of the University hospital where the study was undertaken. None had ever before smoked cannabis in any form, though all had previously smoked tobacco and discontinued the habit at least a year before our study. They had been assessed psychiatrically and were considered objective witnesses with no apparent emotional overlay.

Cigarettes were prepared in the laboratory from one batch of cannabis leaf, grown locally. Each cigarette weighed 1 g and contained approximately 10 mg of tetrahydrocannabinol.[5] Subjects smoked one cigarette, through a holder, over approximately 10 minutes. A double-blind study was planned but abandoned, for our subjects could distinguish dummy "simulated" marihuana cigarettes from the natural product after the first few puffs. Instead, the effects of tobacco smoking were similarly assessed in three subjects one week after investigation of their response to marihuana.

Limb blood flow was measured by venous-occlusion plethysmography.[6,7] Blood pressure was determined by auscultation, and pulse rate from the continuous plethysmographic record. Respiratory rate and depth were recorded through a stethograph, and cardiac activity on an electrocardiogram. Skin and rectosigmoid temperatures were measured by standard thermocouples (Light Laboratories), the skin thermocouple being applied to the nail bed of the great toe and the rectosigmoid probe passed after suitable preparation of the subject. Each figure in Tables 1-4 represents the mean of observations made over a three-minute period.

The investigation comprised three separate series of experiments:

First Series

Cardiovascular effects of cannabis and its influence on the integrity of peripheral vascular reflex responses were studied in six subjects. For the latter purpose, mental and noxious stimuli that normally cause a rapid fall in hand flow were used.[8-10] These were mental arithmetic problems not exceeding three figures and ice placed for five seconds on one hand while blood flow was recorded in the contralateral hand.

Second Series

Similar studies of the effects of tobacco (two French Gauloise cigarettes smoked over 10 minutes)

From the Royal Free Hospital Medical School, Liverpool Road Branch, London N 1, England, where reprint requests should be addressed to Dr. Beaconsfield.

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Table 1. Effect of Marihuana Smoking on Pulse Rate and Cutaneous Temperature.*

Subject No.	Pulse Rate (per min)				Blood Pressure (mm Hg)			Toe Temperature (°C)		
	C†	1	2	3	C	1	2	C	1	2
1	60	82	88	96	118/74	125/85	120/75	24.5	25.6	26.0
2	72	90	92	88	120/80	120/80	125/80	28.5	30.0	29.5
3	74	88	100	92	120/70	135/80	130/78	24.7	27.0	27.5
4	58	84	84	80	110/70	125/80	120/80	27.5	28.0	28.0
5	68	90	92	88	130/75	130/84	135/80	29.0	30.2	30.2
6	64	98	94	90	115/72	140/76	125/75	23.5	25.3	25.5
Mean	66	89‡	92‡	89‡				26.3	27.7‡	27.8‡

*Each figure represents mean of measurements over 3 consecutive min.
†C represents control, 1 immediately after, 2 30 min after, & 3 60 min after smoking completed.
‡Significantly > control mean ($p < 0.001$).

were made in three subjects from the first series one week after they had smoked marihuana.

Third Series

Possible mechanisms responsible for the tachycardia and increased limb flow were studied in four other volunteers, each of whom acted as his own control. Responses to cannabis alone were measured, and one week later marihuana was smoked after either oral propranolol for 48 hours (40 mg every six hours), to block beta-adrenergic vascular activity, atropine (0.6 mg subcutaneously), to induce efferent vagal blocking effect, or infusion of norepinephrine and of epinephrine (10 μg per minute intravenously for 10 minutes).

Subjects were tested at rest after an overnight fast, in standard laboratory conditions. After control observations made over 30 minutes, smoking was begun, and measurements made during smoking, immediately after termination of smoking, and intermittently, for one hour subsequently.

Results

Circulatory Effects of Marihuana Smoking

Pulse. A rise in heart rate was generally apparent before the cigarette was finished. The mean pulse rate immediately after smoking was 89 as compared to 66 per minute in the control period (Table 1). Thereafter, pulse rate remained elevated, increasing further in four subjects, averaging 92 at 30 minutes and 89 at one hour; these values are all significantly different from the control mean.

Table 2. Effect of Marihuana Smoking on Limb Blood Flow.*

Subject No.	Forearm Blood Flow				Calf Blood Flow			Hand Blood Flow		
	C†	1	2	3	C	1	2	C	1	2
	ml/100 ml of tissue/min									
1	6.3	9.6	10.0	10.0	2.1	4.3	4.3	2.9	4.9	3.8
2	4.7	10.0	9.4	6.8	3.5	5.9	3.5	1.9	2.4	2.8
3	4.4	6.5	5.3	6.0	1.2	3.9	2.7	5.2	5.6	5.0
4	4.8	9.0	8.5	7.6	3.6	6.0	4.3	3.9	6.3	3.8
5	6.4	6.8	7.0	4.9	—	—	—	1.4	1.8	1.6
6	5.9	7.5	6.0	5.3	—	—	—	—	—	—
Mean	5.4	8.2‡	7.7‡	6.8	2.6	5.0‡	3.6	3.1	4.6	4.0

*Each figure represents mean of 6 consecutive determinations over 3 min.
†C represents control, 1 immediately after, 2 30 min after, & 3 60 min after smoking completed.
‡Significantly > control mean ($p < 0.02$).

Table 3. Effects of Atropine and Propranolol on Circulatory Responses to Marihuana Smoking.*

Period of Study	Subject No.	Pulse Rate	Blood Pressure	Forearm Blood Flow
		per min	*mm Hg*	*ml/100 ml of tissue/min*
Control	7	68	115/70	6.0
	8	72	125/70	5.5
	9	80	120/75	3.9
	10	82	120/80	4.6
After smoking†	7	92	140/75	9.1
	8	90	120/80	10.2
	9	102	125/85	6.5
	10	98	135/85	8.8
Control	9	84	125/85	4.9
	10	80	130/80	8.4
After atropine†	9	104	135/90	5.2
	10	100	130/85	8.8
Smoking after atropine	9	136	140/100	7.0
	10	128	145/100	12.0
After propranolol§	9	72	125/80	2.9
	10	68	120/75	3.4
Smoking after propranolol	9	76	120/80	3.1
	10	72	118/75	2.7

*Figures represent calculated mean values of measurements made over 3 consecutive min (values for forearm flow derived from 6 readings of blood flow taken at ½-min intervals).
†Immediately after smoking marihuana.
‡½ hr after injection of atropine.
§40 mg/6 hr/2 days.

Electrocardiogram. For technical reasons, standard limb and aVR leads only were taken. In five of six subjects the main change observed during and for about 30 minutes after smoking was increased width and decreased amplitude of the P wave in Lead 2 and inversion of the T wave in Lead 3.

Blood pressure and respirations. Systolic pressure increased slightly in some subjects, but overall there was no significant change (Table 1). Similarly there was no overall change in respiratory rate or excursion in response to marihuana smoking.

Temperature. Cutaneous toe temperature increased significantly from a mean of 26.3°C initially to 27.7°C after marihuana smoking and remained elevated for at least 30 minutes (Table 1). No change was recorded in the temperature of the rectosigmoid region.

Peripheral blood flow. Blood flow increased concomitantly with the rise in pulse rate. There was a significant increase in both forearm and calf flow after the cigarette was finished – 5.4 and 2.6 to 8.2 and 5.0 ml per 100 ml of tissue respectively – but a slight rise only in hand flow (Table 2), from 3.1 to 4.0 ml; 30 minutes later, forearm and calf flow were still elevated, averaging 7.7 and 3.6 ml respectively whereas mean hand flow had fallen to around control levels. Mental arithmetic and ice caused no significant change in hand flow after smoking, whereas before cannabis these stimuli induced the expected reduction in flow (Fig. 1).

Circulatory Effects of Tobacco Smoking

Pulse rate increased on the average 15 beats per minute during smoking but fell to control values

Table 4. Effects of Marihuana Smoking on Responses to Intravenous Norepinephrine and Epinephrine (10 μg per Minute).*

Period of Study	Subject No.	Pulse Rate	Blood Pressure	Forearm Blood Flow
		per min	*mm Hg*	*ml/100 ml of tissue/min*
Control	7	78	110/60	6.6
	8	74	120/75	6.0
	10	82	120/80	3.5
During norepinephrine†	7	68	130/80	3.3
	8	64	135/90	5.0
	10	68	135/90	3.0
Control	7	70	120/70	6.0
	8	72	120/75	5.0
	10	80	125/80	3.8
After smoking‡	7	90	140/75	8.5
	8	100	130/80	7.0
	10	96	130/80	6.1
During norepinephrine†	7	72	130/90	5.4
	8	74	135/90	5.2
	10	78	140/90	4.5
Control	7	72	120/80	2.9
	8	76	115/85	5.4
	10	82	120/80	4.0
During epinephrine†	7	82	140/70	4.5
	8	84	130/70	7.2
	10	98	130/65	6.5
Control	7	72	120/85	3.9
	8	66	135/90	5.7
	10	84	120/75	3.6
After smoking‡	7	88	140/85	5.1
	8	82	130/80	8.2
	10	98	120/75	6.8
During epinephrine†	7	100	115/60	10.2
	8	98	115/50	11.8
	10	116	100/50	9.0

*Figures represent calculated mean values of measurements made over 3 consecutive min (values for forearm flow derived from 6 readings of blood flow taken at ½-min intervals).
†During last 3 min of 10-min intravenous infusion.
‡Immediately after smoking marihuana.

within 30 minutes. Systolic and diastolic pressure rose 10 and 5 mm of mercury respectively for up to 30 minutes.

Forearm and calf flow fell in two subjects and increased in one after smoking; control levels were regained within 30 minutes. The mean change in flow, from 4.8 to 5.1 ml per 100 ml of tissue per minute, was not significant.

Hand flow fell with smoking in all three subjects from a mean of 4.4 ml to 1.9 ml, but averaged 4.0 ml within 30 minutes.

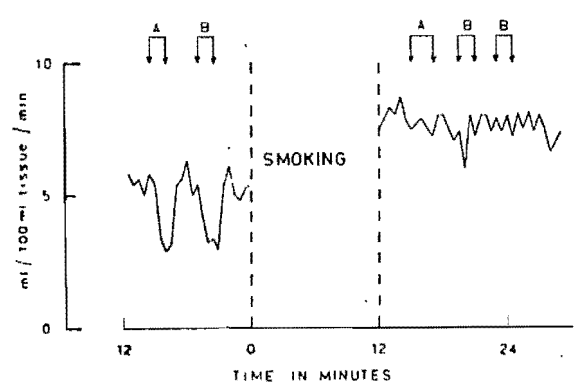

Figure 1. Effect of Mental Arithmetic (A) and Ice Placed on Contralateral Limb (B) on Hand Blood Flow, before and after Smoking Marihuana.

Cutaneous temperature fell with smoking from 25.9 to 23°C and remained at that level for the duration of the experiment. There was no change in temperature of the rectosigmoid region.

Effects of Pharmacologic Agents (Tables 3 and 4)

Responses to marihuana alone were as described in the first series.

Propranolol prevented both the tachycardia and the increase in forearm flow previously evoked by cannabis.

Atropine increased pulse rate but not forearm flow or blood pressure. When cannabis was then smoked, blood pressure increased and cardiac rate rose further, to around 100 beats per minute for more than three hours, whereas in non-atropinized subjects, tachycardia was sustained only up to 90 minutes. In two further volunteers atropine, administered 30 minutes after cannabis smoking, produced an additional elevation in pulse rate and also in blood pressure; both remained significantly raised for three hours.

Epinephrine and norepinephrine initially evoked their characteristic pattern of circulatory response.[11] After marihuana, when the pulse rate and forearm flow had increased, epinephrine induced further cardiac acceleration, to around 100 beats per minute, and a marked rise in forearm flow to a level greater than in the control infusion. Epinephrine also caused a greater fall in diastolic pressure after cannabis than before, and whereas systolic pressure was initially increased by epinephrine, it fell during the infusion given after marihuana smoking. Norepinephrine induced similar responses after smoking as before — a fall in cardiac rate and peripheral flow and a rise in systolic and diastolic pressures.

Discussion

An increase in limb flow accompanying the tachycardia invariably evoked by smoking of marihuana is thus apparent. This peripheral vasodilatation seems to be more marked in arterioles of skeletal muscle than in those of skin, for the rise in forearm and calf flow was greater and more sustained than in the hand. The possibility that circulatory adjustments occur in other vascular beds is suggested by the fact that the increase in peripheral flow was unaccompanied by a fall in systemic pressure and also by some of the changes recorded in the electrocardiogram. That we observed no change in rectosigmoid temperature does not exclude altered flow in other vascular beds after marihuana. A marked reduction in temperature, as a dose-dependent effect, was recorded in rats after cannabis,[12] and a fall in temperature was also observed after excessive doses in man.[13]

Peripheral circulatory responses to tobacco, as reported by Abramson[11] and confirmed in the present study, contrast with those to marihuana. The effect of tobacco differs from that of marihuana in other respects, for tobacco stimulates epinephrine release and increases blood lactate, nonesterified fatty acids and glucose,[14] whereas in studies still in

progress, we found no change in concentration of these metabolites after marihuana.

Although systemic administration of synthetic tetrahydrocannabinol would have been a simpler experiment, this study was designed to obtain pertinent data concerning physiologic effect of cannabis in the context of its customary social use.

Our demonstration that marihuana smoking impairs vascular reflex responses is therefore of particular interest. We did not apply powerful "stress" stimuli; the arithmetical problems were simple, and the ice caused only slight discomfort. Stronger stimuli would undoubtedly cause vasoconstriction in the hand after marihuana, but the fact that hand flow was unaffected by standard stimuli, though mental performance was apparently unimpaired, suggests that marihuana smoking obtunds the body's vasomotor reflex mechanisms. Even in paraplegic patients with a high-cord lesion, ice on the contralateral limb produced a fall in hand flow.[10] Thus, in an emergency, the subject's reflex vascular responses might not be as rapid or as widespread as when he is not under the influence of the drug.

From our use of pharmacologic agents to modify vascular responses evoked by cannabis, it appears that both the tachycardia and the increased muscle flow are mediated by beta-adrenergic vascular mechanisms, for there was no change in cardiac rate or forearm flow after marihuana in the subjects pretreated with a beta-adrenergic blocker. Epinephrine release during cannabis smoking is unlikely to be the exciting stimulus, since, in the presence of a beta-adrenergic blocker, constrictor effects of epinephrine would be unopposed; forearm flow would therefore fall, and blood pressure rise. That we found blood lactate, nonesterified fatty acids and glucose, which are normally increased by epinephrine, to be unchanged after marihuana supports this conclusion.

The tachycardia is unlikely to result from an atropine-like effect on efferent vagal activity, since it was still evoked in subjects who were clinically atropinized. On the contrary, vagal activity may normally limit the degree and duration of cannabis-induced tachycardia, as indicated by the greater and more sustained increase in pulse rate and blood pressure observed after smoking in subjects given atropine — irrespective of whether that alkaloid was administered before or after marihuana.

Our findings have several clinical implications. The age group most frequently involved in road traffic accidents is also the one that most commonly smokes marihuana. A persistently high cardiac rate in a patient in an accident, not adequately explained by the clinical situation, might be related t cannabis smoked before the accident. With hind sight, this hypothesis may explain circulatory distur bance, in a number of accident cases that, at th time, we could not explain. On subsequent ques tioning, some of these patients admitted to smokin marihuana shortly before the accident. Premedica tion with atropine or local anesthetic infiltration containing epinephrine in such patients could en hance and prolong this tachycardia for a dangerous ly long period.

The possibility of altered responses to vasoactiv compounds, particularly those influencing adrener gic and autonomic activity, should thus be borne i mind, and counsels caution in administering suc drugs and anesthetic agents to those who migh recently have smoked marihuana.

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Cardiovascular effects of prolonged delta-9-tetrahydrocannabinol ingestion*

In contrast to the tachycardia and unchanged or increased blood pressure seen after single doses, prolonged delta-9-tetrahydrocannabinol (THC) ingestion produced significant heart rate slowing and blood pressure lowering in hospitalized volunteers. Impaired circulatory responses to standing, exercise, Valsalva maneuver, and cold pressor testing suggest a state of sympathetic insufficiency. Marked weight gain was observed in all subjects, which has been shown to be related to fluid retention and plasma volume expansion. Tolerance developed to orthostatic hypotension, possibly related to plasma volume expansion, but did not develop to the supine hypotensive effects. Nearly complete tolerance developed to the tachycardia and psychological effects produced by smoked marijuana while ingesting THC. Electrocardiographic changes were minimal despite the large cumulative dose of THC. The hypothesis that THC has a biphasic effect on the sympathetic nervous system in man, producing excitation with single doses and inhibition with prolonged administration, is discussed.

Neal L. Benowitz, M.D., and Reese T. Jones, M.D. *San Francisco, Calif. The Langley Porter Neuropsychiatric Institute and the Medical Service, Division of Clinical Pharmacology, San Francisco General Hospital, University of California*

In addition to its psychological effects, marijuana and its primary active ingredient, delta-9-tetrahydrocannabinol (THC), have significant cardiovascular effects in man. The usual cardiovascular response to single doses of smoked, ingested, or intravenous cannabis is increased heart rate with increased or unchanged supine blood pressure.[12, 20, 28] Occasionally, after large single doses, orthostatic hypotension has been noted in man.[10, 19, 22] In light of this observation and the observations that THC lowers blood pressure in experimental animals,[9] some investigators have suggested that marijuana or its derivatives might be useful as antihypertensive agents. There are, however, very few studies that have examined the effect of prolonged cannabis administration on the circulatory system of man. Bernstein and associates[2] have recently reported that hospitalized volun-

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Reprint requests to: Neal L. Benowitz, M.D., Langley Porter Neuropsychiatric Institute, 401 Parnassus Ave., Room A-309, San Francisco, Calif. 94143.

*The findings and conclusions in this report are those of the individual investigators and do not necessarily reflect the position of the National Institute on Drug Abuse.

Table 1. *Cardiovascular measurements before, during, and after prolonged oral THC administra*

Measurements	*Stages*				
	Pre-drug	*Accel.*	*Early maximal*	*Mid-maximal*	*Late maxima*
Supine					
Systolic blood pressure (mm Hg)	115.8	118.6	111.1†	108.2‡	107.9
Diastolic blood pressure (mm Hg)	62.8	60.8	58.9	54.5‡	53.3
Heart rate (bpm)	68.4	70.0	64.3	60.9‡	62.3
Standing—1 min					
Δ Systolic blood pressure (mm Hg)	−9.6	−10.9	−15.5‡	−15.2‡	−11.2
Δ Diastolic blood pressure (mm Hg)	+1.9	+1.5	−2.7	−1.2	+1.5
Δ Heart rate (bpm)	+14.6	+18.5	+21.8	+21.6	+18.1
Exercise—1 min					
Δ Systolic blood pressure (mm Hg)	+13.8	+7.7†	+6.2†	+9.6	+8.5
Δ Diastolic blood pressure (mm Hg)	−6.0	−12.2	−10.9	−9.8	−10.2
Δ Heart rate (bpm)	+5.4	+12.2†	+11.7	+14.7‡	+11.6
Cold pressor					
Δ Systolic blood pressure (mm Hg)	+13.1	+10.2	+9.6	+7.1	+11.1
Δ Diastolic blood pressure (mm Hg)	+13.4	+12.9	+11.8	+14.6	+15.9
Δ Heart rate (bpm)	+13.3	+12.1	+11.1	+12.5	+14.3
Valsalva—Maximum pulse slowing (bpm)	−16.8	−19.3	−15.8	−10.2	−11.6
Valsalva—time to bradycardia (sec)	4.02	4.78	5.85‡	5.82‡	5.6
Body weight (kg)	67.4	68.9‡	69.8‡	70.7‡	71.0

*Three-way repeated measures analysis of variance, df = 5, 40.

†$p < 0.05$
‡$P < 0.01$
} paired comparisons to pre-drug measure, Dunnett post test.

teer subjects who smoked 3 to 6 marijuana cigarettes per day for 21 days were found to have slightly lower systolic and diastolic blood pressures during marijuana smoking.

We have been investigating some of the effects of prolonged high-dose THC administration in man.[13] Because subjects are studied in a controlled hospital environment, we were able to systematically examine circulatory changes that accompany long-term THC ingestion. Specifically, this report attempts to answer the following questions: (1) "What are the cardiovascular effects and possible mechanisms involved?" (2) "Does tolerance develop to these effects?" and (3) "What happens during drug abstinence?"

Methods

The subjects were 12 male volunteers between 20 and 27 (mean ± SD: 25.1 ± 2.2) years of age. Their levels of cannabis use prior to entering the study ranged from 2 to 21 marijuana cigarettes (mean of 9) weekly. The number of years since their first use of cannabis regularly was a mean of 4 (2 to 6) yr. No one with evidence of major psychiatric, cardiopulmonary, or liver disease or a history of frequent heroin, barbiturate, or amphetamine use was included. All subjects were in good health as judged by complete physical and neurologic examination, screening blood and urine tests, chest x-ray, electrocardiographic, and electroencephalographic examinations. Subjects were asked not to use any drugs for one week prior to the study. They were then admitted to the Clinical Research Ward of Langley Porter Neuropsychiatric Institute for 30 days and advised that they could withdraw from the study at any time. They were paid $25.00 per day during the hospitalization.

The longitudinal study was conducted so that each subject was treated and evaluated during 6 consecutive phases: (1) placebo, the first 5 to 7 days; (2) accelerating (increasing) doses of THC, 2 to 4 days; (3) early fixed maximal dose of 210 mg per 24 hr for 5 days; (4) middle maximal (210 mg) dose, next 5 days; (5) late maximal (210 mg) dose, last 4 to 8

in man (12 subjects)

Post-drug	*F**	*P**
116.5	14.66	<0.01
61.2	11.83	<0.01
64.4	6.88	<0.01
−6.5	11.12	<0.01
+7.0†	6.78	<0.01
+13.8	1.90	NS
+11.1	3.10	<0.05
−3.7	2.32	=0.05
+4.2	5.26	<0.01
+11.8	2.19	NS
+16.1	0.79	NS
+14.7	0.86	NS
−14.5	2.65	<0.05
4.56	6.70	<0.01
68.9	20.24	<0.01

days; and (6) placebo, 4 to 6 days immediately following the last maximal dose period. The phases were of variable duration so that neither subjects nor investigators knew exactly when the THC was started and stopped. Subjects received THC for a total of 18 to 20 days. In this experimental design, pre-THC observations serve as a control for each subject. For those parameters that initially change during the period of THC administration but return to pre-THC values thereafter, the presence of a drug effect may be clearly ascertained. For those parameters that do not, the effects of hospitalization itself may not be clearly distinguishable from prolonged drug effects.

The THC was administered orally in gelatin capsules every 4 hr. Each capsule contained zero to 30 mg of purified THC in sesame oil, obtained from the National Institute on Drug Abuse. The exact dose varied according to a predetermined schedule under double-blind conditions. Single capsules were administered every 4 hr except for 12 midnight, when a double dose was administered. The total maximum daily dose for all subjects was 210 mg. At the maximum dose, the dose per kilogram varied from 2.6 to 3.9 mg/kg (mean 3.2 ± 0.4) every 24 hr. Marijuana cigarettes containing 20 mg THC, also obtained from the National Institute on Drug Abuse, were administered periodically in order to evaluate cross-tolerance between oral and smoked marijuana.

Blood samples for various laboratory tests were always obtained in the morning with the subjects still at bed rest. These included weekly complete blood counts, SMA-12 profiles, and on 6 subjects, thrice weekly hemoglobin and hematocrits. Body weight was measured each morning before breakfast on a balance scale with subjects dressed only in underwear. About 2 hr after the 8:00 A.M. dose, a cardiovascular examination was performed that consisted of the tests discussed in the following paragraphs.

Supine blood pressure and heart rate after 10 min of rest. Blood pressure was measured by auscultation with a mercury manometer. Disappearance of the Korotkoff sound was taken as the estimate of diastolic pressure. Heart rate was determined from a 60-sec count of the radial pulse.

Standing blood pressure and heart rate. Blood pressure and heart rate were determined at 1-min intervals while the subject stood quietly for 3 min. In this case, as well as after exercise, the radial pulse was counted for 15 sec at each minute. Blood pressure and heart rate data after standing and exercise are reported both as absolute scores and as change from supine scores.

Blood pressure and heart rate after exercise. Subjects exercised by stepping up and down on a 12-inch platform 36 times in 1 min in time with a metronome, followed by quiet standing for 3 min. Blood pressure and heart rate were recorded immediately after exercise and for each minute for 3 min thereafter.

Blood pressure and heart rate during Valsalva maneuver. Twice a week, subjects in the sitting position were asked to exhale into a mouthpiece connected to a mercury manometer to maintain a pressure of 40 mm Hg for 20 sec. Heart rate was continuously monitored by electrocardiogram, and rates were measured at various phases according to the bedside method

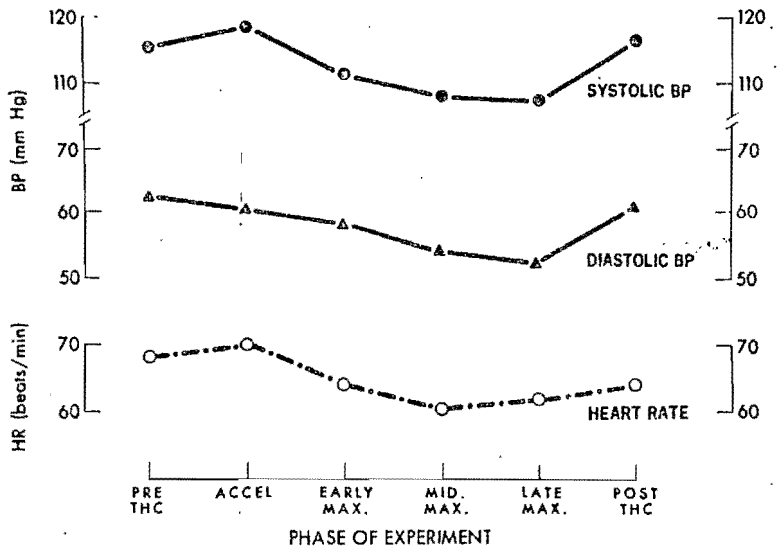

Fig. 1. Supine arterial blood pressure and heart rate during phases of prolonged THC ingestion. Maximum THC dose is 210 mg/day. Total duration of drug ingestion is 18 to 20 days. Duration of phases of the experiment is defined in the text. Each point represents a mean value of 12 subjects. Statistical analysis of this data is presented in Table I.

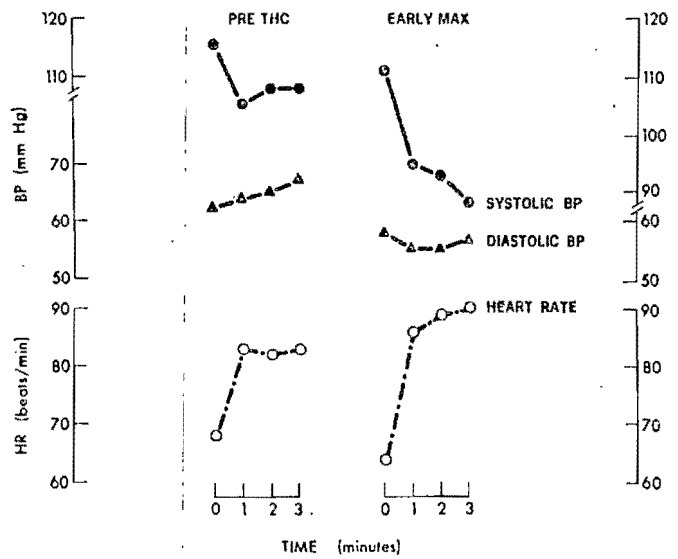

Fig. 2. Time-course of arterial blood pressure and heart rate from supine rest (0 min) to quiet standing for 3 min. *Pre-THC* measurements were taken during placebo phase. *Early max* measurements were taken during the first 5 days on 210 mg THC/day (which had been preceded by 2 to 4 days by increasing doses of THC). Each point represents a mean value of 12 subjects.

described by Elisberg.[6] Heart rate is reported as change from pre-Valsalva heart rate.

Cold pressor test. Twice a week, subjects in the supine position were asked to place one hand to the wrist in ice water for 30 sec. Blood pressure was obtained before and at the end of the immersion period, and heart rate was recorded continuously by electrocardiogram. Data are reported as change before and after cold immersion.

Electrocardiogram. Full 12-lead electrocardiograms were performed with subjects at rest twice a week.

Heart rate response to smoked marijuana.

Heart rate was measured before and after smoking a marijuana cigarette at the end of the pre-THC period, at the end of the period of maximal THC ingestion, and in the post-THC period. Resting heart rate, taken after 20 min of quiet sitting, determined pre-smoking heart rate. The post-smoking rate was measured at the end of a 10 min smoking period.

Plasma volume. Three additional male subjects, whose complete cardiovascular data are not included in this report, were administered THC in a dose schedule similar to that of the 12 subjects composing the basic study group. Plasma volumes were measured by the Evans Blue dye method[8] on two occasions: before THC administration and after 14 days of regular THC ingestion.

Analysis of data. Because of the slight variations in dose schedule among subjects, data were grouped according to the phase of the study as described previously. Data collected from each individual within each phase was averaged to obtain a single mean value for that subject in that phase. The means of phases were treated then as single observations and compared for significant difference as a repeated measure in a 3-way analysis of variance. Absolute and change scores were analyzed separately. For this analysis, subjects were also divided into groups according to: (1) frequency of marijuana use (frequent users, 15.2 ± 5.3 joints per week vs less frequent users, 4.7 ± 2.2 joints per week); and (2) by dose per body weight of THC administered (high dose, 3.49 ± 0.29 mg/kg vs low dose, 2.82 ± 0.9 mg/kg). Paired comparisons between pre-THC and other stages were performed using a Dunnett post test.[5]

Results

Cardiovascular changes for selected variables at different phases of the study are shown in Table I.

Supine blood pressure and heart rate. The changes while on drug were compared to baseline values. There was no significant change in resting blood pressure or heart rate in the acceleration phase of drug administration, although one subject demonstrated hypertension and tachycardia (pre-THC: 114/68, pulse 60 to 154/84, pulse, 86). This was associated with a sensation of internal hyperactivity and anxiety. As THC administration was continued at the 210 mg dose level through phases 3, 4, and 5, a small but significant fall in systolic blood pressure (7.9 mm Hg, $p < 0.01$), diastolic blood pressure (9.5 mm Hg, $p < 0.01$), and decrease in heart rate (7.5 bpm, $p < 0.01$) developed, as shown in Fig. 1. When THC was discontinued, systolic and diastolic pressures returned to normal values within one day, while heart rate remained slightly but not significantly slower than the pre-drug value.

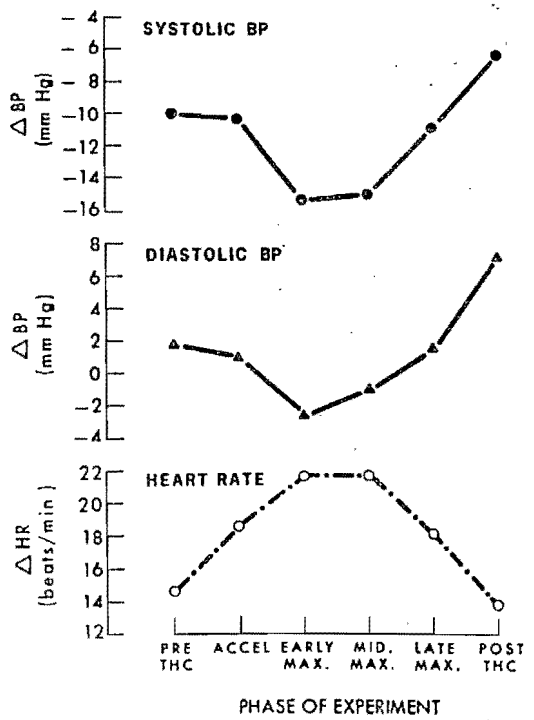

Fig. 3. Change in arterial blood pressure and heart rate from supine to quiet standing for 1 min during phases of THC ingestion. Maximum THC dose is 210 mg/day. Total duration of drug ingestion is 18 to 20 days. Duration of phases of the experiment is defined in the text. Each point represents a mean value of 12 subjects. Statistical analysis of these data is presented in Table I.

Response to standing. Before THC administration, systolic and diastolic blood pressures decreased slightly after standing but then stabilized coincident with a small increase in heart rate. During the period of THC ingestion, the systolic and diastolic pressures fell significantly more than in pre-drug periods and did not stabilize over 3 min, as shown in Fig. 2. At the

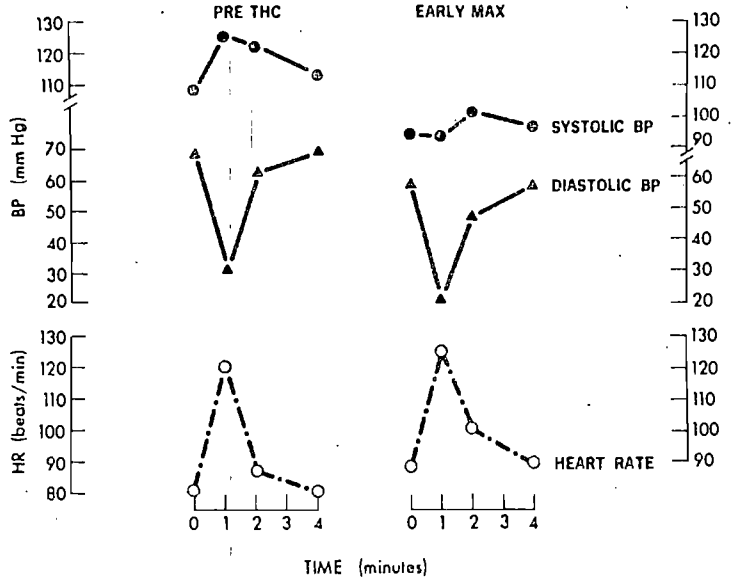

Fig. 4. Time-course of arterial blood pressure and heart rate before and after upright exercise (of 1 min duration). Zero-min measurements were made during relaxed standing; 1-min measurements immediately after completion of exercise; 2- to 4-min represent the 3 min after completion of exercise. *Pre-THC* measurements were taken during placebo phase. *Early max* measurements were taken during the first 5 days on 210 mg THC/day (which had been preceded by 2 to 4 days by increasing doses of THC). Each point represents a mean value of 12 subjects.

time of maximal postural hypotension (early maximal phase), blood pressure fell to frankly hypotensive levels (111/59 supine to 95/56 standing) with only a modest compensatory increase in heart rate (64 to 86). Seven of the subjects experienced marked postural dizziness at this stage. In 2 cases during phase 3, subjects were unable to stand still in the erect posture for 3 min without syncope. Changes in blood pressure and heart rate after 1 min standing are depicted at different stages of the study in Fig. 3. Postural hypotension was most marked during the early maximal drug dosage and became less evident as the drug was continued. Postural changes at 2 and 3 min after standing are not shown, but follow a similar pattern over the duration of the experiment.

Response to exercise. The cardiovascular responses immediately after and for 3 min after exercise are illustrated in Fig. 4. During the pre-drug phase, subjects had the expected increase in the systolic blood pressure and heart rate with a marked decrease in diastolic blood pressure, all of which returned to baseline levels within 2 min. During early phases of THC administration, subjects demonstrated both lower baseline blood pressures and blunting of the usual systolic blood pressure increase. Peak heart rate response immediately after exercise in the early maximal THC period was comparable to the response in the pre-THC period, despite mean blood pressures that fell to much lower levels during THC. Two subjects were unable to perform the exercise in the early maximal dose period because of dizziness. As with other cardiovascular effects, the exercise-related blood pressure response after one minute standing was most abnormal during early drug periods and less prominent with continued drug. As with supine blood pressures and response to standing, the cardiovascular responses to exercise returned to pre-drug values within one day after THC was discontinued.

Valsalva maneuver. Heart rate slowing following the release of Valsalva is a parasympathetically mediated response to the blood pressure overshoot that normally occurs at this time.[6] The degree of heart rate slowing was significantly impaired while subjects were ingesting maximal doses of THC (pre-THC

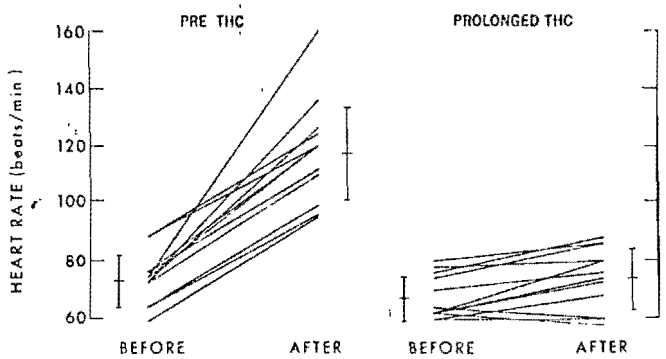

Fig. 5. Heart rate before and immediately after smoking a marijuana cigarette containing 20 mg THC in 12 subjects. *Pre-THC* values were obtained in placebo phase. *Prolonged THC* values were obtained after 14 or more days of THC ingestion. Bars adjacent to heart rate values represent mean ± SD.

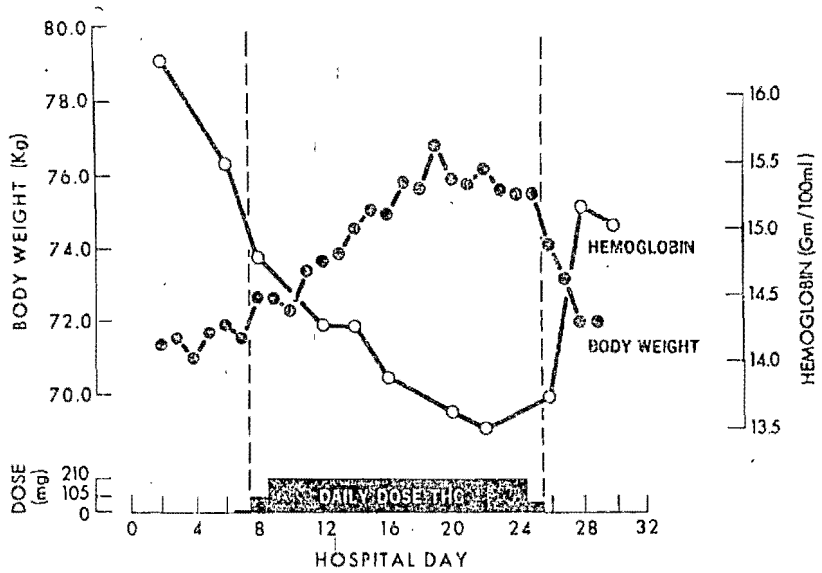

Fig. 6. Time-course of body weight and hemoglobin concentration during prolonged ingestion of THC. Daily dose of THC is shown by the black bar graph. Dashed lines represent days when THC was started and stopped. Each point represents the mean value for 6 subjects. Note that body weight increases as THC is started, and abruptly declines after THC is discontinued. Hemoglobin follows an inverse course.

heart rate decreased 16.8 bpm, mid-maximal THC decrease, 10.2 bpm, $p < 0.01$). In addition, the time from end of Valsalva to onset of bradycardia was delayed (4.02 to 5.82 sec, $p < 0.01$). There was no significant impairment of the heart rate increase during sustained straining.

Cold pressor test. Blood pressure response to immersion of the hand in ice water (data shown in Table I) suggests ($0.05 < p < 0.10$) impairment of systolic pressure response during THC administration. This was most marked in the mid-maximal THC phase and gradually returned to baseline with continued drug administration.

Electrocardiogram. Heart rate slowing was noted (Table I) in the routine electrocardiograms performed at biweekly intervals during the THC study. One subject demonstrated two junctional premature beats and one premature beat, which could have been either junctional or ventricular in one record while ingesting

THC. Five subjects demonstrated slight changes in P wave axis during THC ingestion, 3 of which returned to pre-drug axis after THC was discontinued. A slight change in T wave axis, seen as inversion of T waves in Lead III or becoming upright in Lead aV_L, was noted in 3 of the subjects, one of which also had P wave axis changes. All T wave changes returned to pre-THC patterns. There were no significant changes in P-R, QRS, or Q-T intervals, other than those related to rate changes.

Heart rate response to marijuana cigarettes. As shown in Fig. 5, all subjects experienced heart rate increase after smoking a marijuana cigarette in the pre-THC period (mean heart rate increase was 44.6 ± 13.5). This increase was significantly reduced after prolonged THC ingestion (6.6 ± 6.9; paired comparison, $t = 8.95$, $p < 0.01$). There was a similar decrease in subjective effects that accompanied the diminished heart rate effect. Responsiveness to both heart rate and subjective effects returned to pre-drug levels when patients were re-examined 1 wk after THC was discontinued.

Body weight and plasma volume. Mean body weights in various phases of the study are shown in Table 1. Mean weight increase was 3.6 kg ($p < 0.01$). Daily body weights and periodic blood hemoglobin concentrations are shown for 6 of the 12 subjects who were on the same daily schedule of THC in Fig. 6. Body weight increased progressively from the beginning of THC administration until about the tenth day on drug. Immediately after THC was discontinued, body weights precipitously fell, approaching pre-THC levels within 3 to 4 days. Changes in hemoglobin concentration were inversely related to changes in body weight, suggesting that the increased body weight was associated with increased plasma volume. The differences between pre-THC and post-THC hemoglobin concentrations probably reflect blood losses of approximately 150 cc per week associated with repeated venipunctures.

Plasma volume increased in all three subjects where this was measured, from a mean of 43.7 ± 2.0 ml/kg to 51.3 ± 3.0 ml/kg after 14 days of THC ingestion. The proportion of change in plasma volume to change in body weight was 20.7 ± 1.6%, which is comparable to the proportion of plasma volume to extracellular fluid volume.

Drug history and THC dose. No significant differences in cardiovascular changes associated with THC ingestion were noted between subjects with high vs low marijuana use history or between subjects with high vs low THC dose per kg body weight.

Discussion

Cardiovascular effects and possible mechanisms. The data presented indicate that there is significant blood pressure lowering and heart rate slowing in normal subjects during prolonged ingestion of THC. The effects of THC administration on cardiovascular responses to standing, exercise, Valsalva, and cold pressor were examined to gain information about mechanisms of THC effects on cardiovascular reflexes and to evaluate functional capacity during chronic administration.

Postural adjustments are normally mediated by the carotid baroreceptor, producing sympathetically mediated arteriolar constriction and cardiac stimulation.[25] Orthostatic hypotension in the absence of hypovolemia and associated with submaximal compensatory heart rate increase after THC suggests a state of sympathetic insufficiency, resembling that seen after treatment of patients with sympatholytic drugs like guanethidine.[17]

With exercise in normal man, sympathetically mediated cardiac stimulation and skeletal muscle venoconstriction serve to increase cardiac output, while total systemic vascular resistance is reduced due to arteriolar dilatation.[25] During THC administration, the usual systolic blood pressure increase is impaired, while the usual diastolic pressure fall occurs, thereby causing a marked decrease in mean blood pressure. The degree of heart rate increase is insufficient to return the blood pressure to normal levels. Sympathetic inhibition involving the heart and peripheral veins is suggested, although direct THC-mediated venodilatation could account for part of the response.

Delayed and diminished heart rate slowing after release of Valsalva are consistent with sympathetic inhibition, but could also be seen

as a result of impaired venous return secondary to direct drug-induced venodilatation or impaired cardiac output secondary to drug-induced myocardial depression.[6] The effect of THC on the pressor response to immersion of the hand into cold water was quite variable among subjects, but also suggests a diminished response, consistent with sympathetic inhibition.[26]

Weight gain was seen in all subjects, and in 3 additional subjects expansion of plasma volume was documented. The proportion of plasma volume to total body weight increase suggests that weight gain was due to extracellular fluid expansion. This is most likely a compensatory response to chronic blood pressure lowering, as is well documented for patients receiving sympathetic-blocking antihypertensive medications.[27]

Most studies of the cardiovascular effects of smoked or ingested cannabis in man have described tachycardia with unchanged or slightly increased supine blood pressure.[12, 20, 28] The heart rate and blood pressure increase after THC administration, which is associated with increased limb blood flow, is blocked by propranolol, suggesting that THC initially produces beta sympathetic excitation.[1] Occasionally, postural hypotension has been described in man after smoking or ingesting THC,[10, 19, 22] but has not been systematically studied. Other studies in man have demonstrated (1) impaired reflex venoconstriction after deep breathing following oral THC,[28] (2) diminished cold-induced reduction in hand blood flow after smoked marijuana,[1] and (3) suppressed cardiac slowing after Valsalva maneuver after smoked THC,[23] all of which are consistent with our observations during THC ingestion. In anesthetized animals, hypotension and bradycardia have been observed after large intravenous doses of THC.[9] Animal studies suggest that THC may inhibit central nervous system mediated sympathetic discharge as the mechanism for the cardiovascular effects.[9]

The cardiovascular responses to THC in man appear to be biphasic. The published data with single-dose studies suggest sympathetic excitation. Our data from studies of prolonged oral doses and other previously published data after large single doses suggest sympathetic inhibition, although the possible contributions of either direct vasodilatation or myocardial depression cannot be excluded. To the extent that data from anesthetized animals can be applied to man, central nervous system sympathetic inhibition must be considered as a possible explanation for the effects we have observed.

The observation of sustained supine and standing blood pressure decreases in man suggests possible applicability of THC as an antihypertensive medication. Although the blood pressure effects in these normal volunteers were small and measured over only 2 to 3 wk of THC administration, one cannot predict what the effects on a hypertensive subject might be. The maximum dose administered in this study was well tolerated by most subjects and may not reflect the upper limits of a dose for use in man. Further studies are indicated to determine if THC or congeners thereof may be of use as a hypertensive medication in man.

The electrocardiographic changes during prolonged ingestion of THC were of special interest in light of changes previously noted after single doses.[1, 12, 14, 24] In our study, nonspecific P or T wave changes were noted in 7 subjects while taking THC, although in no case did the overall record appear abnormal. These findings, which are similar to those reported after single doses, occurred promptly after administration of THC, did not change substantially while continuing THC, and in most cases reverted to pre-THC patterns promptly when THC was discontinued. Occasional premature beats were noted in 2 of the subjects; 1 of the beats may have been ventricular in origin. In a report of the effects of prolonged marijuana smoking, minor repolarization changes and occasional premature atrial or ventricular contractions were reported in a few of the subjects.[2] The findings of our study of prolonged oral THC and of the study of prolonged smoked THC mentioned above[2] suggest that THC has minor effects on resting electrocardiograms. However, since the electrocardiograms were recorded over brief time periods, the question of whether THC increases the incidence of premature ventricular contractions cannot be evaluated.

Observations of tolerance and abstinence. The cardiovascular data demonstrate that tolerance to the supine hypotensive effects does not develop in this study period, but does develop to the orthostatic hypotension and associated symptoms. One possible mechanism in development of such tolerance appears to be the expansion of plasma volume. The observation that body weight stabilized during the last week on THC suggests that the specific hypotensive stimulus to compensatory volume expansion persists without the development of tolerance. A striking observation is the development of marked tolerance to the tachycardia produced by smoked marijuana as a result of prolonged ingestion of oral THC. Bernstein and associates[2] have reported that heavy marijuana smoking in a controlled hospital environment is associated with the development of some tolerance to heart rate and psychological effects, but many other investigators have not documented tolerance in casual or hospitalized smokers.[21, 23] In casual use and in most studies of heavy marijuana smoking, subjects smoke marijuana cigarettes only during waking hours, so that the night provides time for recovery of sensitivity. Our subjects, in contrast, receive oral THC, which is absorbed much more slowly than inhaled THC, every 4 hr both day and night, so that consistent THC effects are produced. In this circumstance, tolerance is most likely to develop.

The dose of THC given these subjects is larger than usually consumed by most American users, but not beyond what is smoked or ingested in India, Afghanistan, or Jamaica.[3] A good-quality American marijuana cigarette contains 5 to 20 mg THC. Smoked THC is estimated to be 3 or 4 times as potent as oral THC.[11] Thus, in comparison, the subjects in the study by Mendelson and associates,[18] who selected their own dose of about six 18 to 23 mg THC cigarettes per day, were ingesting equivalent amounts of THC as subjects in this study. Despite the large dose, our 12 subjects tolerated the administration of cannabis without difficulty. During the first week of drug administration, they appeared sedated, lethargic, and sluggish with poor concentrating ability, slowed speech and motor activity, and ataxia. After 18 to 20 days of cannabis administration, almost complete tolerance to the sedative and other intoxicating effects had occurred, so that subjects performed normally in a variety of motor and psychological tests. After discontinuation of THC, a mild abstinence syndrome, characterized predominantly by insomnia, anorexia, irritability, and sweating, was described by several patients. Details of the behavioral and physiological effects during prolonged oral cannabis ingestion and in abstinence are reported elsewhere.[13]

Abstinence syndromes after marijuana use have been anecdotally reported in man in Indian users and in Rhesus monkeys after chronic high-dose cannabis administration,[4, 7] but have not been well characterized. We took special note, therefore, of the cardiovascular changes in THC on the immediate days after discontinuation of the drug. There was no rebound hyperactivity, and cardiovascular parameters returned to pre-drug levels within 1 day after THC was discontinued. This suggests that the cardiovascular effects of THC are dependent on continual high levels of THC or some active metabolite. Tissue accumulation, which has been suggested by findings of concentration of THC in adipose tissue[15] and by the long metabolic half-life,[16] appears not to be playing an important role in the cardiovascular effects.

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Δ^9-Tetrahydrocannabinol: Effects on Mammalian Nonmyelinated Nerve Fibers

Abstract. *Δ^9-Tetrahydrocannabinol can be applied to tissue in vitro by dissolving it in Pluronic F68 and ethanol. It causes a decrease in size of the compound action potential of the nonmyelinated fibers of the vagus nerve of the rabbit. This effect appears to be dose-related and chloride-dependent. Effects on other measurable parameters of nerve function seem to be minimal. Although the amounts required seem to be higher than those required to produce hallucinogenic effects in man, this effect is consistent with other work on Δ^9-tetrahydrocannabinol and may ultimately account for a significant portion of the pharmacological activity of this drug.*

Despite a great interest in the pharmacological properties of marihuana constituents there is almost no information about the effects of these substances on elemental nervous tissue. This lack of information is largely the result of the difficulty in dissolving the tetrahydrocannabinols in nontoxic aqueous media. We report here first, a method of dissolving Δ^9-tetrahydrocannabinol (Δ^9THC) in an innocuous medium, and second, a well-defined effect of Δ^9THC on nervous conduction.

Δ^9-Tetrahydrocannabinol is not readily soluble in water, which presents a major difficulty in studies of this compound on isolated tissues. It can be carried into solution by Tween 80 (*1*), but the high concentrations of the detergent required (up to 2 percent) are themselves toxic to many preparations. In the experiments reported here, which examine the effect of Δ^9THC on mammalian nonmyelinated nerve fibers, the difficulty has been overcome through the use of Pluronic F68 (*2*). This is a high molecular weight surfactant compound that has general applications in bringing into solution compounds which are otherwise insoluble in water; for example, the present experiments suggested its use (successfully) in studies of axon fluorescence with a relatively water-insoluble merocyanine dye (*3*). A small amount of Pluronic (100 mg) was dissolved (by gentle heating to less than 60°C) in 0.15 or 0.2 ml of ethanol containing 15 mg of Δ^9THC (*4*). The resulting solution could be readily dissolved in 100 ml of Locke solution (*5*) to produce a 500 μM solution of Δ^9THC. This solution, which was initially clear, became increasingly cloudy over the next few hours, as if some colloidal suspension of Δ^9THC were being formed. No analytical determinations of the amounts of Δ^9THC in solution were made. All experiments to test solutions of Δ^9THC were started within half an hour of preparation.

A desheathed cervical vagus nerve from a rabbit, killed by injection of air into an ear vein, was mounted either for monophasic electrical recording in the sucrose gap apparatus (*6*), or for diphasic recording in a glass capillary chamber (1 mm in diameter) in which five annular platinum electrodes for stimulation and recording were embedded (*7*). The compound action potential of the nonmyelinated fibers was elicited every minute; the conduction distance was 10 to 25 mm. The temperature was about 20°C. Changing the perfusion fluid from normal Locke solution to one containing Pluronic F68 (100 mg/100 ml) and ethanol (0.2 ml/100 ml) produced little or no effect on the size of the compound action potential (or on the size of the spike in the giant fibers of lobster and squid used in later experiments). However, as Fig. 1 shows, changing to a solution that also contained 500 μM Δ^9THC produced a fall in the height of the compound action potential of 15.2 ± 1.6 percent (mean ± standard error, 12 experiments) by the end of 30 minutes. About half of the experiments were done with monophasic recording, and the other half with diphasic recording. No significant difference was seen in the two groups, so the results of all experiments were treated as a single population. In four experiments a similar exposure to 100 μM Δ^9THC produced an average fall of 12.9 ± 3.7 percent, and in four experiments an exposure to 30 μM Δ^9THC produced an average fall of 8.7 ± 2.8 percent. In three experiments 10 μM Δ^9THC produced a fall of 7.3 ± 1.7 percent. In two experiments 3 μM Δ^9THC produced just visible responses of 0 and 2.8 percent.

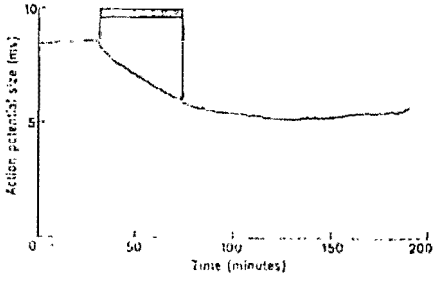

Fig. 1. Effect of 500 μM Δ^9THC, applied to the nerve during the interval between the arrows, on the size of the monophasic compound action potential of the nonmyelinated fibers of the desheathed rabbit cervical vagus nerve at 21.1°C.

The effect (Fig. 1) continued to develop during the time of exposure to the drug (up to 1 hour). In seven experiments with 500 μM Δ^9THC the average size of the compound action potential fell by 7.5, 13.3, and 18.6 percent of its initial value after exposures to the drug of 15, 30, and 45 minutes, respectively. In experiments in which the exposure to Δ^9THC was further continued, the fall after 1 hour (four experiments) was about twice, and after 4 hours (one experiment) four times, that obtained after a 30-minute exposure. When the Δ^9THC was removed and the nerve was returned to Locke solution containing just Pluronic F68 and ethanol, the height of the compound action potential always stopped declining. It usually remained constant as long as it was subsequently observed (30 to 60 minutes), although on the rare occasions when it was followed for 1 to 2 hours (as in Fig. 1), some late recovery toward the pre-Δ^9THC values sometimes did occur. A similar delayed recovery from the tetrahydrocannabinols, still incomplete after many hours, had already been observed in experiments in which the guinea pig ileum was stimulated transmurally (*8*).

The reduction in size of the compound action potential seemed to be accompanied by a slowing in conduction velocity. This effect was, however, small. In five experiments a 30-minute exposure to 500 μM Δ^9THC caused the conduction velocity to fall by 2.7 ± 0.8 percent. However, since a 30-minute exposure to normal Locke solution was itself accompanied by a slight decrease in conduction velocity the true effect of Δ^9THC was smaller, being 1.8 ± 0.9 percent. There was no marked or consistent effect on the electrical threshold, nor was there any evidence that the sodium pump was affected, 500 μM Δ^9THC producing no great change in the time constant of decay of the post-tetanic hyperpolarization recorded with the nerves in a Locke solution in which

75 to 100 percent of the chloride had been replaced by isethionate (*9*).

Complete replacement of the chloride of the Locke solution by the presumably impermeant anion isethionate led to a marked reduction in the effect of Δ^9THC. In six experiments a 30-minute exposure to 500 μM Δ^9THC produced a decrease of 17.2 ± 2.5 percent in the height of the compound action potential when applied in normal Locke solution containing chloride; in similar experiments on paired nerves from the same rabbits carried out in chloride-free Locke solution the fall was only 5.8 ± 1.7 percent.

These experiments demonstrated that Δ^9THC directly affects nerve fibers, an action that is particularly interesting in the light of the reported absence of any action on ganglia (*10*). It is important to establish which of the fundamental properties of the nerve membrane is altered by the Δ^9THC to produce the effects seen. All fibers might be affected, the sodium conductance, for example, being turned off earlier, or the potassium conductance being turned on earlier; either of these effects, by shortening the duration of the elemental spikes in each elemental single fiber, would reduce the size of the compound action potential. Alternatively, some particular Δ^9THC-sensitive fibers may be blocked completely. Unfortunately, experiments done to examine these possibilities with internal electrodes—on giant axons of lobsters, in which the maximum rate of rise of the spike was measured, and of squid, in which the sodium and potassium currents in voltage-clamp experiments were measured—proved negative. But because of the preliminary nature of these experiments we cannot say whether the apparent lack of effect of Δ^9THC on marine invertebrate axons reflects a true absence of action on these axons.

Quantitatively similar results to those just described were also obtained with a water-soluble THC analog, ADL 1137 (*11*). In concentrations of 100 to 500 μM this drug produced a fall in the size of the compound action potential of the nonmyelinated fibers of the rabbit vagus nerve of about the same size as that produced by Δ^9THC. Furthermore, the water-soluble analog shared with Δ^9THC the ability to reduce the size of the compound action potential of the myelinated fibers of this nerve. The myelinated fibers may prove more suitable for a voltage-clamp examination of the action of Δ^9THC, in which the effects on the sodium and potassium currents of the nerve membrane can be examined, and such studies are now under way.

We have not yet determined the minimum concentration of Δ^9THC required to produce the kind of effect described here when applied for 1 to 2 hours or more. Threshold effects were obtained with half-hour exposures to 3 μM Δ^9THC, which is equivalent to 1 mg/liter. At first sight, this seems a good deal higher than the psychoactively effective doses in humans, which are 50 to 250 μg/kg (*12*). However, the experimental conditions are quite different, so the two values are not really comparable.

A more relevant comparison might be based on the amount of Δ^9THC actually taken up by the brain when given intravenously in animals. Furthermore, the opportunity for biotransformation or metabolism of the Δ^9THC is probably more limited in the nerve in vitro than in vivo. If the effects of Δ^9THC depend to some extent on the production of active metabolic products, as has been suggested (*13*), it might not be appropriate to compare the concentrations used in the present experiments with psychoactive doses in man. It should be noted that the proposed active metabolite 11-OH-Δ^9THC showed, in a single experiment, qualitatively similar effects on the vagal nonmyelinated fibers to those just described. Furthermore, the psychologically inactive compound, cannabinol, was similarly found to be inactive on nerve.

An effect of THC on the conduction system was postulated by Lapa *et al.* (*14*) on the basis of experiments in which they recorded potentials from the sensory nucleus of the trigeminal nerve. The present experiments provide direct confirmation of this suggestion. And an action of the sort described here on the fine nonmyelinated terminals of neurons and dendrites might explain some of the many effects of this drug on nervous tissue.

The question must certainly arise: Are these results on isolated nerve at this dosage pertinent to the known pharmacological effects of marihuana in man? Unfortunately, dosage per se may not be relevant, since the rabbit vagus nerve may resemble the human nervous system only insofar as both share the same basic physiological mechanisms of conduction. The sensitivity of different parts of nervous systems to drugs may vary considerably, and it may well be that some parts of rabbit or human nervous systems are far more sensitive than our selected test object. Even the minimum concentrations used in these experiments are at least an order of magnitude greater than those reported in human plasma during peak psychedelic action (*15*). This means that the significance of these results for understanding the human response is at the moment unclear. However, this is the first demonstrated pharmacological effect of marihuana constituents on a simple nerve preparation, and it would seem to provide at least a starting point in working out a mode of action.

R. Byck

Department of Psychiatry and
Department of Pharmacology,
Yale University School of Medicine,
New Haven, Connecticut 06510

J. M. Ritchie

Department of Pharmacology,
Yale University School of Medicine

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The Inhibition of DNA Synthesis by Cannabinoids[1]

R. A. Carchman, L. S. Harris, and A. E. Munson

Department of Pharmacology, Medical College of Virginia, Virginia Commonwealth University, Richmond, Virginia 23298

SUMMARY

Several of the cannabinoids found in marihuana have been shown to inhibit tumor growth and increase the life-span of mice bearing the Lewis lung adenocarcinoma. When trypsin-dispersed isolated Lewis lung cells are incubated *in vitro*, they maintain their capacity to carry out macromolecular synthesis (RNA, DNA, protein). This process can be inhibited by cytosine arabinoside, actinomycin D, or methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea, whereas cyclophosphamide, an agent that must be bioactivated, was inactive. Inhibition of DNA synthesis as measured by [^{3}H]thymidine uptake into acid-insoluble material was used as an index of cannabinoid activity against isolated Lewis lung cells, L1210 leukemia cells, and bone marrow cells incubated *in vitro*. Δ^9-, Δ^8-, 1-hydroxy-3-*n*-pentyl-, and 1-Δ^6-*trans*-tetrahydrocannabinol, and cannabinol demonstrated a dose-dependent inhibition of DNA synthesis whereas cannabidiol and 1-hydroxy-3-*n*-pentylcannabidiol were markedly less inhibitory in our *in vitro* cell systems. Furthermore, our *in vitro* observations with these cannabinoids are supported by *in vivo* tumor inhibition studies. Ring modifications as in cannabichromene or cannabicyclol abolish *in vitro* activity as does dihydroxylation at the 8β and 11 positions of 1-Δ^9-*trans*-tetrahydrocannabinol. Δ^9-*trans*-tetrahydrocannabinol demonstrated the least toxicity of all inhibitory cannabinoids *in vivo*; this is supported by its lesser effect on bone marrow DNA synthesis *in vitro*.

INTRODUCTION

Recently, we reported (5, 6, 12) that certain cannabinoids exhibit inhibitory activity against the Lewis lung adenocarcinoma *in vitro* and *in vivo* and that the Friend leukemia virus induced splenomegaly *in vivo*. This report expands the *in vitro* observations to include additional cannabinoids and other compounds using L1210 leukemia cells, isolated mouse bone marrow cells, and trypsin-dispersed isolated Lewis lung cells. The spectrum of effects attributed to Δ^9-THC,[2] the major psychoactive ingredient in marihuana, is varied and complex (17). To date, an understanding of any of its behavioral or biochemical effects is still in the realm of speculation. Recent investigations aimed at elucidating the cellular responses to marihuana or Δ^9-THC have shown that cultures of human lung cells show changes in DNA synthesis following exposure to marihuana smoke (9). Zimmerman and McClean (22) demonstrated in *Tetrahymena* that exposure to Δ^9-THC produced cytolysis that was preceded by the inhibition of RNA, DNA, and protein synthesis. Aberrant chromosomal changes have been reported in chronic marihuana users (21) as well as impairment of spermatogenesis and testosterone secretion (8). Mitogen-stimulated lymphocytes from marihuana users have been reported to incorporate [^{3}H]thymidine at a lower rate than did lymphocytes from control subjects (13). These observations suggest a complex interaction between the drug and the various systems studied. The investigations in our laboratories are currently directed toward the identification of active cannabinoids (structure activity relationships); proposal of structural modification of the THC ring system that alter activity; and elucidating their mechanism(s) of action. This report deals primarily with *in vitro* structure activity observations.

MATERIALS AND METHODS

Preparation of Isolated Lewis Lung Cells. Lewis lung tumors were grown in the gluteus muscle of C57BL/6 mice. Tumors were removed 14 to 18 days posttransplant, cleared of tissue debris, and cut into 1- to 2-mm sections. They were resuspended in MEM (with Earle's salts) containing 20% heat-inactivated fetal calf serum. Cells were enumerated using a Model ZB1 Coulter counter diluted in Isoton, and cell viability was monitored using trypan blue dye exclusion (0.5% trypan blue). Cells were centrifuged (600 × *g* for 10 min) and resuspended in MEM (with Earle's salts) supplemented with (for every 500 ml MEM) 5 ml 100× vitamins, 10 ml 50× amino acids, 5 ml 200 mM glutamine, 5 ml penicillin (5000 units/ml)-streptomycin (5000 μg/ml). Tissue culture reagents were obtained from Grand Island Biological Co., Grand Island, N. Y., or Flow Laboratories, Rockville, Md. Cell number was adjusted to 10^7 cells/ml and dispensed in 25-ml Erlenmeyer flasks (3 to 5 ml/flask) containing 10 μl of drug or drug vehicle (ethanol). Flasks were then allowed to equilibrate for 15 min at 37° under an atmosphere of 5% CO_2-95% O_2 with shaking in a Dubnoff metabolic bath. Radiolabel was then added and 1-ml aliquots for liquid scintillation were removed at various times. The quality of cells that one can isolate depends upon the care with which one chooses the tumor and its trypsinization. The use of old

[1] This investigation was supported in part by grants from HEW(DA 00490, CA17840, CA17551), the Alexander and Margaret Stewart Trust Fund, and an American Cancer Society Institutional Grant (IN105A).

[2] The abbreviations used are: Δ^9-THC, 1-Δ^9-*trans*-tetrahydrocannabinol; THC, *trans*-tetrahydrocannabinol; MEM, minimal essential medium; ara-C, 1-β-D-arabinofuranosylcytosine; ED_{50}, 50% inhibition of cell proliferation; Δ^8-THC, 1-Δ^8-*trans*-tetrahydrocannabinol; ABN Δ^8-THC, 1-hydroxy-3-*n*-pentyl-Δ^8-tetrahydrocannabinol; CBD, cannabidiol; ABN-CBD, 1-hydroxy-3-*n*-pentylcannabidiol; CBN, cannabinol.

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necrotic tissue is undesirable as are extended periods of trypsinization (>2 hr). It became apparent following repeated use of the Erlenmeyer flasks that acid wash followed by alcohol-acetone rinses was insufficient in removal of cannabinoid material from the glassware. We therefore have been using 20-ml glass scintillation vials which are then discarded.

Isolation and Incubation of Mouse Bone Marrow Cells. C57BL/6 × DBA/2 F_1 (hereafter called B6D2F_1) mice were sacrificed (cervical dislocation) and the tibias and fibulas were freed of surrounding muscle and connective tissue. The distal portions of bones were removed and 1 ml heparinized MEM (1.0 unit/ml) was forced through the bone using a 1-ml syringe with a 26-gauge needle. Cells were then centrifuged (600 × *g* for 10 min), resuspended 3 times in MEM, and the nucleated cells were enumerated using a Coulter counter. Cell viability was monitored by trypan blue dye exclusion. Cell number was adjusted to 10^7 cells/ml and incubated with the drug or drug vehicle as described above.

Isolated L1210 Cells. DBA/2 mice were inoculated with 1 × 10^5 L1210 cells 6 days prior to sacrifice (cervical dislocation). L1210 cells were removed by flushing the peritoneal cavity with 10 ml unsupplemented serum-free Dulbecco's MEM containing penicillin and streptomycin. Cells were centrifuged (600 × *g* for 10 min) and resuspended in the above serum-free medium at a final concentration of 10^7 cells/ml. L1210 cells were then incubated as described for Lewis lung cells.

Radiolabel Uptake *in Vitro*. Radiolabeled [*methyl*-^{3}H] thymidine (6.7 Ci/mmole; New England Nuclear, Boston, Mass.) and/or [2-^{14}C]uridine (57 mCi/mmole; New England Nuclear) were added (10 μCi/flask) following the 15-min equilibration period of cells with drug or drug vehicle. At various times after the addition of radiolabel, 1-ml aliquots were removed and placed in 12- x 75-mm test tubes containing 2 ml 10% trichloroacetic acid (4°). The samples were mixed and then kept at 4° for at least 20 min prior to filtration on a Millipore filtering apparatus. The filters were washed 3 times with 3 volumes of 10% trichloroacetic acid (4°). Filters were then transferred to glass scintillation vials and 10 ml of toluene-Liquiflor cocktail were added. Samples were counted in a Beckman liquid scintillation spectrometer. The incorporation of radioactive precursors into acid-insoluble material (DNA) was linear over the incubation period (45 min) for Lewis lung, L1210, and bone marrow cells.

Drugs. ara-C (NSC 63878), actinomycin D (NSC 3053), methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (NSC 9544), and cyclophosphamide (NSC 26261) were kindly supplied by Dr. Ruther Geran, National Cancer Institute. Cannabinoids were supplied by Dr. Monique Braude of the National Institutes of Drug Abuse or Dr. Raj Razdan of The Sheehan Institute for Research, Cambridge, Mass.

RESULTS

Effect of Drugs on [^{3}H]Thymidine Uptake. In an attempt to evaluate our *in vitro* systems, we tested compounds of known activities. ara-C, an inhibitor of DNA synthesis (3), has been shown to be highly active against the L1210 *in vivo* (13) and is extremely active against the L1210 *in vitro* (Table 2A). Lewis lung or bone marrow cells incubated *in vitro* are apparently not as sensitive to this agent (Table 2A); this is supported by its marginal activity against the Lewis lung tumor *in vivo* (Table 1). At nontoxic doses of ara-C (10, 40 mg/kg) significant inhibition of primary tumor size was seen only at 14 days; at later times *i.e.*, 21 and 28 days, no significant inhibition of the primary tumor was observed. Significant increases in the life-span of these animals was seen only at the highest nontoxic dose used (40 mg/kg). Actinomycin D, an inhibitor of RNA synthesis (14–16), when incubated with Lewis lung cells *in vitro* in the presence of radiolabeled thymidine and uridine, inhibits only the uptake of [^{14}C]uridine into acid-precipitable material while not affecting [^{3}H]thymidine uptake. Inhibition of DNA synthesis by actinomycin D was only seen when uridine incorporation was less than 30% of control (data not shown). Methyl-1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea is a purported alkylating agent (11) that showed only marginal activity in our *in vitro* system against the Lewis lung, and cyclophosphamide which is an alkylating agent requiring bioactivation (2) showed no activity when tested against the L1210 *in vitro* (data not shown).

Effect of Cannabinoids and ara-C on [^{3}H]Thymidine Uptake *in Vitro* and *in Vivo* Tumor Inhibition. We tested several cannabinoids in our *in vitro* systems as indicated in Table 2A and 2B. Similar data (not shown) were also obtained using radiolabeled uridine. Those cannabinoids that inhibited [^{3}H]thymidine uptake by 50% (ED_{50}) in the Lewis lung *in vitro* at μM concentrations include Δ^9-, Δ^8-, ABN Δ^8-THC and CBN (Table 2A). Concentrations of cannabinoids in excess of 10^{-5} M were required, for CBD, ABN-CBD, cannabichromene, 8β, 11-dihydroxy, Δ^9-THC, and cannabicyclol (Table 2B). Comparisons of *in vitro* activity for several of these compounds with *in vivo* tumor inhibition can be made by comparing Tables 2 and 3. Dose-dependent inhibition of primary tumor growth is seen with those compounds that inhibited *in vitro* DNA synthesis at μM concentrations (Table 2A). Those compounds (CBD, ABN-CBD) that required larger concentrations *in vitro* either were inactive or as is seen with CBD stimulated tumor growth at either 25 or 200 mg/kg (Table 3). Significant (>25%) increases in the life span of Lewis lung tumor-bearing mice were seen only with Δ^9-, Δ^8-, ABN Δ^8-THC, and CBN (data not shown). The inhibition of DNA synthesis in the L1210 *in vitro* is depicted in Table 2. A comparison of cannabinoid activity in this system is of interest since basically the activities fall into the same groups seen with the Lewis lung. Although Δ^9-THC has an ED_{50} greater than Δ^8-THC, ABN Δ^8-THC, or CBN, it is at least 10 times more effective than the other cannabinoids (Table 2). The cannabinoids have not been shown to have any activity against the L1210 *in vivo* (11) and, if one compares the best *in vitro* activity against any of the cannabinoids (CBN) with ara-C, a drug that can cure the L1210 *in vivo*, there is approximately a 100-fold difference in their ED_{50}'s. Comparison of cannabinoid activity against normal bone marrow cells *in vitro* is seen in Table 2. Δ^9-THC was the only cannabinoid that inhibited both Lewis lung DNA synthesis *in vitro* and tumor growth *in vivo* while

Table 1

The effect of ara-C in Lewis lung adenocarcinoma in vivo

Groups of B6D2F$_1$ male mice were inoculated i.m. with 5×10^5 Lewis lung tumor cells. ara-C was administered i.p. daily for 10 consecutive days beginning 24 hr after tumor implantation.Tumor weights were calculated from caliper measurements by:

$$\frac{w = a \times b^2}{2}$$

where a is the long axis (mm), b is the short axis (mm), and w is weight (mg).

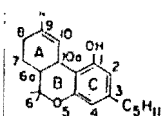
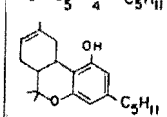
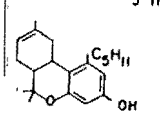
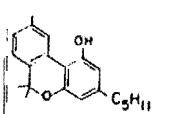
Dose (mg/kg)	Tumor wt (mg) (posttransplant) Day 14	Day 21	Day 28	% increase in life-span
0	2700 ± 200[a] (8)	7000 ± 300 (8)	9600 ± 400 (7)	
10	1600 ± 100 (7)	5500 ± 300 (7)	10000 (1)	0
40	1400 ± 60 (8)	6500 ± 500 (8)	9700 ± 600 (5)	25.4
80		Toxic		

[a] Mean ± S.E. of the tumor weights was derived from the number of mice indicated in parentheses.

Table 2A

The effect of cannabinoids and ara-C on in vitro DNA synthesis

Cells were prepared and incubated with drug or drug vehicle as described in "Materials and Methods." The drug dose that produces ED_{50} of DNA synthesis was calculated using the method of Litchfield and Wilcoxin (10). Values for which an ED_{50} was calculated had slopes that were not significantly different except for ara-C and represent the 15-min incubation time. Drugs were made up fresh daily in ethanol (10 μl/flask) and preincubated with cells for 15 min prior to the addition of radiolabel [^{3}H]thymidine, (10 μCi/flask). Following a 15-min incubation of cells with [^{3}H]thymidine the amount of trichloroacetic acid-insoluble (DNA) radioactivity present was: 25,000 cpm/10^7 cells, Lewis lung; 20,000 cpm/10^7 cells, L1210; 6,000 cpm/10^7 cells, bone marrow.

Drug	Structure	ED_{50}(M) Lewis lung	L1210	Bone marrow
Δ^9-THC		4.18×10^{-6}	3.26×10^{-5}	2.06×10^{-5}
Δ^8-THC		2.99×10^{-6}	8.70×10^{-6}	1.26×10^{-6}
ABN Δ^8-THC		1.48×10^{-6}	5×10^{-6}	3.56×10^{-6}
CBN		2.3×10^{-6}	2.2×10^{-6}	3.08×10^{-7}
ara-C		1.36×10^{-7}	2.53×10^{-8}	1.57×10^{-7}

exhibiting a differential effect on bone marrow DNA synthesis. This apparent selective toxicity was also seen with ara-C [bone marrow *versus* L1210, although not Lewis lung *versus* bone marrow (Table 2A)].

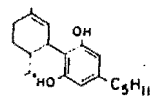
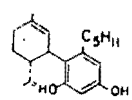
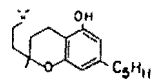
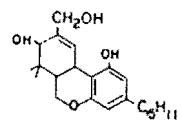
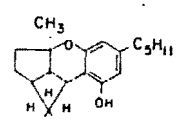
DISCUSSION

The use of isolated cell systems *in vitro* as models to stu and evaluate both basic biochemical and drug mechanisr

Table 2B

The effect of cannabinoids and ara-C on in vitro DNA synthesis

Cell incubations were prepared as described in Table 2A and "Materials and Methods."

Drug	Structure	ED_{50} (M)		
		Lewis lung	L1210	Bone marrow
CBD		3.37×10^{-5}	"	4.89×10^{-4}
ABN-CBD		9.28×10^{-5}	1.29×10^{-2}	5.51×10^{-6}
Cannabichromene		$>10^{-4}$	$>10^{-4}$	
8β, 11-Dihydroxy Δ⁹-THC		$>10^{-4}$	$>10^{-4}$	
Cannabicyclol		$>10^{-4}$		

[a] Doses of CBD (10^{-4} to 10^{-7} M) produced marked stimulation of [^{3}H]thymidine uptake into acid-precipitable material.

Table 3

The effect of cannabinoids on Lewis lung adenocarcinoma in vivo

Groups of B6D2F$_1$ male mice were inoculated with 5×10^5 Lewis lung carcinoma cells in the right hind gluteus muscle. Cannabinoids were administered by gavage daily for 10 consecutive days beginning 24 hr after tumor implantation. Tumor inhibition was determined 14 days after tumor transplant by converting caliper measurements to mg of tumor.

Drug	Tumor wt at the following doses (mg/kg)		
	0	25	200
Δ⁹-THC	2000 ± 240[a]	850 ± 300	700 ± 200
Δ⁸-THC	2200 ± 200	1200 ± 140	1100 ± 150
ABN Δ⁹-THC	2400 ± 130	1400 ± 100	1200 ± 140
CBN	1300 ± 150	1000 ± 150	300 ± 100
CBD	2800 ± 200	4200 ± 500	3900 ± 300
ABN-CBD	2200 ± 200	1900 ± 250	2000 ± 150

[a] Mean ± S.E. derived from 8 mice/group.

represents an important investigational tool. The antitumor activity of Δ⁹-THC reported by Munson *et al.* (12) is consistent with our *in vitro* observations. In addition, all those cannabinoids that we have studied *in vitro* have demonstrated a high degree of correlation when tested *in vivo* (Table 2 *versus* Table 3). We have also evaluated several drugs currently used in treating human leukemia (14). ara-C has been shown significantly to increase the survival time c mice bearing the L1210 leukemia, and indeed its activity i isolated L1210 cells incubated *in vitro* clearly supports its *i vivo* inhibition. When Δ⁹-THC was evaluated *in vitro* agains the L1210, inhibition of DNA synthesis was observed, a though it was less than what was seen with Lewis lung cell incubated *in vitro*. When Δ⁹-THC was tested *in vivo* agains the L1210 leukemia, it showed no activity (12). This appa ent discrepancy may be accounted for by the fact tha agents that significantly prolong the survival time of L121 mice produce significant daily cell kills (>99.9%) (18–20) The data in Table 2A would indicate that Δ⁹-THC is at least to 3 orders of magnitude less potent in inhibition of [^{3}H]thy midine uptake than is ara-C in the L1210. The rapid doub ling time of L1210 cells *in vivo* (0.55 day), which have a com paratively short G_1 phase relative to their S phase, suggest that Δ⁹-THC may not work by inhibiting the S phase of th cell cycle. The kinetics of cell growth (4, 11) in Lewis lun tumor cells *in vivo* (doubling time, 1.7 days for 100 mg o tumor) appear to make this solid tumor more amenable t control by Δ⁹-THC [Munson *et al.* (12)] than is the L121 leukemia and indicates that Δ⁹-THC may work by inhibitin some other phase of the cell cycle. This possibility is cur rently under investigation. ara-C is approximately 10 time less potent in blocking DNA synthesis in the Lewis lung *i vitro* as it is against the L1210. This finding is supported b the observation that this agent is only marginally effectiv against the Lewis lung *in vivo*. These observations suppor the validity of our *in vitro* models in evaluating the cannabi noids. It would also appear from the data on other chemo

therapeutic drugs that these *in vitro* sytstems may provide an appropriate milieu in which drug and biochemical studies can be carried out with a reasonable prediction of their *in vivo* activities.

Following the recent reports of Δ^9-THC activity on lymphocytes (7, 13), testosterone levels (8), chromosome damage (21), and the inhibition of the Lewis lung tumor and Friend leukemia virus-induced splenomegaly *in vivo*, we have attempted to identify the active sites on the THC molecule, to elucidate its sites of action, and to develop more potent analogs. The data from our *in vitro* systems and *in vivo* studies clearly indicate that there is no relationship between central nervous system activity and antitumor properties, since CBN and ABN Δ^8-THC are active *in vitro* and *in vivo* (12) against the Lewis lung while they do not produce any significant behavioral responses (1, 17). The location of the double bond in the A-ring (Δ^9-THC, Δ^8-THC, CBN) does not change its antitumor potency, although we have not evaluated 1-Δ^3-*trans*-tetrahydrocannabinol or other A-ring-saturated derivatives. Substitution or ring alterations on the A-ring are not compatible with activity as seen following hydroxylation (8β, 11-dihydroxy, Δ^9-THC) or alteration of the A-ring as seen with cannabichromene or cannabicyclol. The opening of the B-ring is also incompatible with activity (CBD, ABN-CBD) and in fact appears to increase [^{3}H]thymidine uptake *in vitro*. This structural change also increases the rate of tumor growth *in vivo* and decreases the life-span of the animals with tumor (12). Exchanging the alkyl and phenolic-hydroxyl groups in the C-ring appears not to alter the potency of THC (Δ^8-THC, ABN Δ^8-THC, Table 2A). General conclusions can therefore be made from our *in vitro* observations: (*a*) A-ring constituents are important for activity; this may be related to the planarity of the molecule although other physicochemical changes in the A-ring may provide a further understanding of A-ring requirements; (*b*) the integrity of the B-ring appears essential since CBD and its abnormal derivative show no inhibitory properties; (*c*) modification of the C-ring (ABN Δ^8-THC) does not alter activity. Additionally, these agents have a wide therapeutic index and toxic effects appear to be less serious with cannabinoids than they are with standard chemotherapeutic agents.

The preliminary finding that isolated bone marrow cells are not as drastically affected by Δ^9-THC as the isolated Lewis lung tumor cells represents a significant observation that we are currently pursuing. The lack of such specificity in the other active cannabinoids is supported by the weight loss noted in animals bearing the Lewis lung tumor treated with these drugs (12). In addition, it has been found (J. Levy, personal communication) that peripheral leukocyte counts from Δ^9-THC-treated animals (200 mg/kg), although initially depressed, recover and return to control levels within 4 days. We have also found (unpublished observation) that the total number of peripheral leukocytes from tumor-bearing mice treated with Δ^9-THC (25 to 200 mg/kg) for 10 days are identical to those of untreated animals. We are currently evaluating differential WBC counts from Δ^9-THC animals in order to rule out a change in a specific cell type.

An attempt to understand how the active cannabinoids produce their effects is currently under investigation. Macromolecular (DNA, RNA, protein) synthesis appears to be equally depressed by Δ^9-THC (unpublished data) although this is not related to cell death as indicated by cell viability (>95%). We have studied the action of Δ^9-THC on Lewis lung tumor cells grown in tissue culture; [^{3}H]thymidine uptake studies indicate that drug (Δ^9-THC) concentrations that inhibit thymidine uptake into acid-insoluble material by 50% do not affect the radioactivity in the acid-soluble pool. This observation indicates that Δ^9-THC is not inhibiting DNA synthesis by depressing precursor uptake (A. White and R. Carchman, unpublished observations) in Lewis lung tumor cells.

Our *in vitro* systems for evaluating and understanding the potential chemotherapeutic efficacy of cannabinoids may provide an additional mechanism not only for understanding the activity of these agents but also for accelerating the numbers and kinds of compounds that can be tested. In addition, the isolated bone marrow cells afford an opportunity to extend *in vitro* observations and offer some potential for predicting drug toxicity *in vivo*.

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Toxicity testing *in vitro*. I. The effects of Δ^9-tetrahydrocannabinol and aflatoxin B_1 on the growth of cultured human fibroblasts

JAMES T. COOPER AND SAMUEL GOLDSTEIN

Departments of Pathology and Medicine, McMaster University, Hamilton, Ont., Canada L8S 4J9

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The acute toxicity of Δ^9-tetrahydrocannabinol (Δ^9-THC) and aflatoxin B_1 to two strains of cultured human fibroblasts has been studied. Δ^9-THC had no effect on cell plating efficiency or on the growth of mass cultures at doses of 1 μg/ml (3.18 μM) or less; at 10 μg/ml plating efficiency was reduced by approximately half and at 20 μg/ml colony formation was zero. Aflatoxin B_1 reduced plating efficiency at dose levels of 0.1 μg/ml (0.32 μM) and above; in mass cultures it retarded growth at 1 μg/ml and produced complete inhibition at 5 μg/ml. The potential usefulness of cultured human fibroblasts in toxicity testing is discussed and the importance of using normal diploid cells rather than aneuploid permanent lines is emphasized. The limitations of cell cultures in assessing toxicity, and possible solutions to these are considered.

Introduction

The assessment of toxicity in agents to which man may be exposed is of prime importance. However, there is no wholly satisfactory method for determining toxic limits, since ethical and practical considerations preclude such studies in man himself. The classical approach to this problem is the use of animal studies, although it is difficult to extrapolate from the laboratory animal to man because of interspecies and even interstrain differences. The interpretation of animal data is further complicated by the dependence of the results on animal age and sex, the route and vehicle used for administration, and animal diet (Weisburger 1971). These factors, coupled with the time required to obtain useful results plus the high cost of maintaining animal colonies, combine to make the investigation of alternative methods of toxicity testing attractive.

The use of cultured cells as an *in vitro* system for assaying toxicity has been explored by a number of workers (*e.g.* Metcalfe 1971; Carr and Legator 1973). However, most of the published studies are based on permanent cell lines, often of nonhuman origin. These cells no longer possess the normal diploid chromosomal complement of the original donor and have acquired the ability to grow indefinitely in tissue culture. Conversely, normal diploid cells in culture have a limited lifespan (Hayflick 1965). This disparity in behaviour, together with differences in nutritional requirements (Swim 1967), implies fundamental differences which make permanent lines of limited value in metabolic studies where information about the intact normal organism is desired. Furthermore, the limited *in vitro* lifespan of normal diploid cells, which has been shown to be inversely related to the age of the donor for human skin fibroblasts (Goldstein *et al.* 1969; Martin *et al.* 1970), offers an additional advantage to their use in toxicity testing. It is a useful parameter in terms of which the chronic effects of low doses of an agent can be monitored and yet still provide information within a matter of months.

In this preliminary investigation we have studied the effects of Δ^9-THC and AB_1, on two fibroblast strains derived from normal adults. THC is the principal psychoactive constituent of marihuana (Mechoulam *et al.* 1970) and is of interest in view of the increasingly widespread use of cannabis preparations. It has been studied previously in tissue culture (Zimmerman and McClean 1973; Huot and Radouco-Thomas 1974; Nahas *et al.* 1974), in attempts to elucidate its pharmacological actions but little information is available about its toxicity. AB_1 is a potent mycotoxin with well-documented acute and chronic toxic properties (Wogan and Pong 1970), and was chosen as a control substance to establish the response of the cells to a known toxic agent.

ABBREVIATIONS: Δ^9-THC, Δ^9-tetrahydrocannabinol; AB_1, aflatoxin B_1; RGM, regular growth medium; DMSO, dimethyl sulfoxide; uv, ultraviolet.

Methods

THC (95%, lot SSC 69961, from Health and Welfare Canada, Ottawa, Ont.) was stored at −70 °C in absolute ethanol at a concentration of 5 mg/ml. A stock solution was prepared immediately before use by adding 0.1 ml of the ethanol solution to 4.9 ml of RGM (Eagle's medium supplemented with 15% fetal calf serum (Goldstein and Littlefield 1966)), resulting in a concentration of 100 μg THC per millilitre and 2% ethanol by volume. This was diluted with RGM to the desired final THC concentration. Corresponding solutions containing alcohol alone were used to establish the effects of alcohol on cell growth.

Since THC is relatively unstable, preliminary experiments were carried out to determine its stability under our incubation conditions. For this purpose a solution containing 50 μg THC per millilitre was prepared in glass equipment. Duplicate 5-ml aliquots were extracted immediately and a series of further 5-ml aliquots distributed among plastic petri dishes. Duplicate dishes were extracted immediately and then at various time intervals over the next 6 days following incubation at 37 °C. The extraction procedure employed involved protein precipitation by the addition of 15 ml methanol followed by four successive extractions of the supernatant with 5 ml hexane. The pooled hexane extracts were evaporated to dryness under nitrogen at room temperature and the residue dissolved in 5 ml absolute ethanol. The uv spectrum was recorded on a Pye Unicam spectrophotometer and compared with that of the pure THC suitably diluted from the stock alcohol solution. When a more sensitive gas chromatography – mass spectrometric method for the assay of THC subsequently became available (Rosenfeld *et al.* 1974), this experiment was repeated using a THC concentration of 0.01 μg/ml in RGM.

AB_1 (Calbiochem, CA, U.S.A.) was bought as the purified solid and stored in chloroform solution at 2.5 mg/ml at −70 °C. As required, 0.1 ml was evaporated to dryness, the AB_1 redissolved in 0.25 ml DMSO and 2.25 ml RGM added, producing a final concentration of 100 μg AB_1 per millilitre and 10% DMSO by volume; subsequent dilutions were made with RGM alone. Effects due to DMSO alone were monitored using corresponding solutions prepared in the absence of AB_1.

The two cell strains used were established from skin biopsies of healthy adult males by standard procedures (Cooper and Goldstein 1973), and stored in liquid nitrogen. Ampoules of each at early passage level were thawed into petri dishes (Falcon Plastics, Los Angeles, CA, U.S.A.) and grown to confluence at 37 °C in an atmosphere of 95% air – 5% CO_2. At confluence the cells were rinsed with phosphate buffered saline and harvested with 0.125% trypsin. They were then plated at low (250 or 500 cells per dish) or high (80 000 cells per dish) density into 5-cm petri dishes containing RGM with the appropriate concentration of drug or vehicle. Cells plated at low density were fed again with fresh medium containing drug or vehicle after 7 days and then at the end of 14 days the cells were fixed with formalin and stained with a standard azure–eosin dye (Giemsa's stain) for easy visualization. The number of macroscopically visible colonies was counted to determine the plating efficiency, *i.e.* the fraction of the cells which had survived to form colonies. Triplicate dishes of cells plated at high density for each level of drug and vehicle were harvested daily and the number of cells per dish counted in a Cytograph 6302 cell counter (Biophysics Instruments, Mahopac NY, U.S.A.). This was continued until growth ceased as a result of density dependent inhibition or drug toxicity

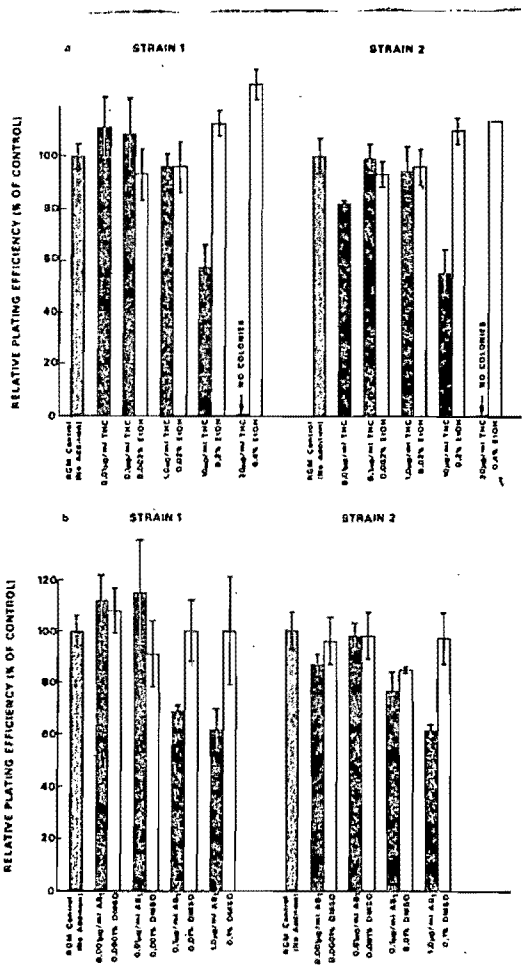

FIG. 1. Relative plating efficiencies of cultured human fibroblasts incubated with (*a*) Δ^9-THC and (*b*) AB_1. Plating efficiency = (No. of macroscopic colonies × 100)/(No. of cells plated). Data are expressed as the percentage of the plating efficiency of the same strain in growth medium alone (mean ± SD).

Results

In the experiment to determine THC stability using the uv spectrum (absorption maxima at 276 and 283 nm, log ε = 3.2 and 3.1, respectively), we found no significant change in extractable THC from zero time up to 6 days at 37 °C using 50 μg THC per millilitre in RGM, with the recovery in the range 72–76%. More importantly, this result was confirmed when the

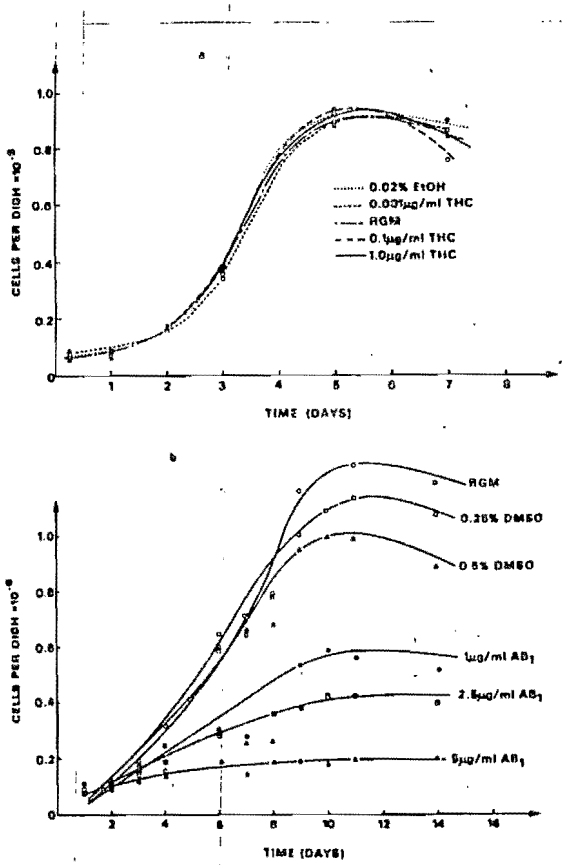

FIG. 2. Mass culture growth of cultured human fibroblasts in the presence of (*a*) Δ^9-THC and (*b*) AB_1.

experiment was repeated with gas chromatography – mass spectroscopic assay of the extracted THC. The specificity of this technique leaves no doubt that Δ^9-THC and not some other moiety is being assayed while its sensitivity permitted the use of a THC concentration in the range used for the studies with cells, *i.e.* 0.01 μg/ml.

The effects of the drugs on colony formation after plating at low density are shown in Fig. 1. At THC concentrations of 0.01, 0.1 and 1.0 μg/ml (0.0318, 0.318 and 3.18 μM, respectively), the plating efficiencies were essentially the same as the RGM control cells which were exposed to neither THC nor alcohol. At 10 μg THC per millilitre (31.8 μM), the plating efficiency of both strains was reduced by almost 50%, while at 20 μg/ml (63.6 μM), colony formation was totally inhibited. These differences were significant (by Student's t test) with $p < 0.005$. Cultures with 0.002% and 0.02% ethanol were not significantly different from the vehicle-free control but the plating efficiencies were elevated in the controls containing 0.2% alcohol ($p < 0.025$) and 0.4% ($p < 0.01$). AB_1 produced no effect at 0.001 or 0.01 μg/ml (0.00318 and 0.0318 μM) but reduced plating efficiency in both strains at 0.1 and 1.0 μg/ml (0.318 and 3.18 μM) with $p < 0.005$; corresponding levels of DMSO alone had no effect.

Figure 2 summarizes data for one of the two strains on the growth of mass cultures. Neither THC at concentrations up to 1 μg/ml nor the corresponding alcohol levels produced any detectable change in growth rate or final cell density during a 7-day period, by which time density dependent inhibition of growth had occurred. AB_1 at 1.0 and 2.5 μg/ml produced progressively increasing inhibition and at 5 μg/ml there was essentially total inhibition of growth. DMSO levels corresponding to 5 and 2.5 μg/ml of AB_1 resulted in some growth inhibition compared to the untreated cells but this was small relative to that produced by AB_1. Similar results were obtained with the second cell strain.

Discussion

Our data with AB_1 are in general agreement with previous studies using permanent lines (Gabliks *et al.* 1965; Harley *et al.* 1969), although the dose level at which we are observing toxic effects, 0.1–1.0 μg/ml, is somewhat lower. This could be due to higher sensitivity of human diploid cells to AB_1 or to slight differences in methodology.

In particular, our results are in good agreement with those of Legator (1969) who also used human cells: he reported growth inhibition of human embryonic lung cells at 0.05 μg AB_1 per millilitre. In additional experiments, not reported in detail, we showed that the same plating efficiency results were obtained if the cells were plated initially into RGM alone and then fed again 24 h later with medium containing the drug or vehicle. This confirms that the reduced plating efficiencies observed were due to a real impairment of cell growth and not merely to reduced attachment of the cells to the dish in the presence of the drug.

Although THC has been reported to be unstable at ambient temperatures, our results confirm that under the incubation conditions used there was no detectable change in concentration over a period of 6 days. We conclude

that oxidation of THC is negligible, probably due to its stabilization by binding to serum protein present in the RGM (Wahlqvist *et al.* 1970).

Serum levels of THC, at which psychoactivity is demonstrable in man, have been established to lie in the region of 10–30 ng/ml (Agurell *et al.* 1973), two orders of magnitude less than the lowest dose producing an acute toxic response in our culture system. However, it may be that long term exposure of cells to THC at levels below those at which acute effects are observed can still produce deleterious effects. This is of importance in view of reports that THC tends to accumulate in fat and brain tissue (Kreuz and Axelrod 1973) and that cell-mediated immunity is depressed in chronic marihuana users (Nahas *et al.* 1974). In an experiment to investigate this, we grew the same strains of cells in mass culture in the presence of various dose levels of THC and AB_1 until they became senescent. Significant reduction of the replicative lifespan of the cells occurred only at the same drug levels that diminished plating efficiency. Consequently, for these two drugs, no additional sensitivity to their toxic effects was demonstrable on chronic exposure over a period of approximately 5 months. The toxicity of THC has previously been studied in animals (Thompson *et al.* 1973*b*; 1973*a*), but direct comparison with our data is difficult, not only because of marked interspecies differences, but also because the THC was given orally and no serum levels were reported.

Our data show that using cultured human fibroblasts to estimate the acutely toxic levels of a known toxic agent, AB_1, we obtain results quantitatively similar to those from other test systems. The results are available both rapidly and relatively inexpensively. Furthermore, the same test system is readily extended to the study of chronic toxicity by observation of the effects of an agent on the overall *in vitro* lifespan of the cells. This may be of value in predicting the effects of long-term exposure to drugs or environmental agents since the limited *in vitro* lifespan of diploid cells represents an excellent model of human aging (Hayflick 1974).

The procedure described is subject to two possible limitations. The drug under study must be solubilized in an aqueous medium, and this in general requires the use of a vehicle. While drug-free controls should clearly be run concomitantly it is still possible that synergistic effects between drug and vehicle may occur and will be ascribed to the drug alone. One means of controlling for this may be to use two chemically dissimilar vehicles whenever possible. Of more specific importance is the fact that many biologically active agents exert their effects through metabolites, in particular, solubilized products of liver oxidation. Although there is evidence that mammalian fibroblasts possess inducible microsomal mixed function oxidase activity (Nebert and Gelboin 1968), this remains to be demonstrated for human diploid fibroblasts. Hence, toxicity due to metabolites might go undetected. A possible solution is the use of a combined test system using purified liver microsomes in conjunction with cultured cells and we report such an approach in detail in a separate paper (Cooper and Goldstein 1976).

Acknowledgment

This research was supported by a grant through the Programme of Research on Drug Abuse co-sponsored by Health and Welfare Canada and the Medical Research Council of Canada and was carried out during the tenure of an Ontario Heart Foundation Senior Research Fellowship (JTC) and a Medical Research Council of Canada Scholarship (SG). Dr. Jack Rosenfeld is thanked for the gas chromatography – mass spectroscopy assay of THC and Diane England for technical assistance.

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Cannabinoids Inhibit Testosterone Secretion by Mouse Testes in vitro

Abstract. *Addition of delta-9-tetrahydrocannabinol or cannabinol to an incubation medium containing decapsulated mouse testes caused a significant reduction in the accumulation of testosterone in the medium. This result suggests that the reported effects of cannabis on male sexual and reproductive function may result from direct inhibition of testicular steroidogenesis by both psychoactive and nonpsychoactive constituents of marihuana.*

Marihuana and its psychoactive constituent, delta-9-tetrahydrocannabinol (THC), have been implicated in the alteration of testicular function in several species including man. Administered through various routes, either marihuana or THC can reduce the concentration of testosterone (T) in peripheral plasma in both rat and man (*1*, *2*), suppress spermatogenesis and produce changes in sperm-head proteins (*3*, *4*), and reduce the weight of the testes and the accessory reproductive organs (*3*–*5*). A reduction in certain androgen-dependent behavioral responses, such as intra- and interspecies aggression (*6*) and copulatory behavior in male rats and mice (*7*), has also been observed. In men, reduced sexual potency and gynecomastia (*1*, *2*, *8*) have been reported in heavy marihuana users. However, changes in aggressive and sexual behavior induced by cannabinoids may be related to the action of these compounds on higher brain centers rather than to changes in the function of the hypothalamic-pituitary-testicular system.

Cannabis-related decreases in peripheral luteinizing hormone (LH) and prolactin levels (*1*, *2*, *9*) suggest that alteration in testicular function may be secondary to suppression of the pituitary. Increased adrenal weight (*5*) and corticosterone production (*10*) with cannabis treatment suggest another possible mechanism for the alteration in testicular function.

In contrast, the demonstration of an inhibitory effect of THC, cannabinol (CBN), and other cannabinoids on the synthesis of protein and nucleic acid in incubated testicular slices (*11*) suggest that THC may act directly on the testis. We therefore studied whether these cannabinoids are capable of directly affecting the testicular biosynthesis of T in vitro. We examined the effects of THC and CBN (which is believed not to be psychoactive) using decapsulated mouse testes in an in vitro incubation system.

Adult (2 to 3 months of age) or immature (34 to 37 days) closed-colony but noninbred laboratory mice were killed by cervical dislocation; the testes were immediately removed, decapsulated, and incubated in Krebs-Ringer bicarbonate buffer, glucose (1 mg/ml), and 12.5 × 10^{-3} international unit of human chorionic gonadotropin (Follutein, Squibb) per milliliter (*12*). The THC or CBN, at the various doses, was introduced into the incubation medium in a 20-μl volume of ethanol. The same amount of ethanol was added to the control flasks. The concentration of T in the medium after 4 hours of incubation was determined by radioimmunoassay (*13*) after suitable dilution of the aliquot (*14*). As a control, mouse testes were incubated either with ethanol (at doses of 10, 20, or 50 μl/ml) or without ethanol. At these doses, ethanol did not affect T release. The differences between the mean T concentration in alcohol-containing and in control incubations were no greater than 9 percent and were not significant.

The effect of THC on testes obtained from adult mice is shown in Table 1. The

Table 1. Effects of treatment with Δ^9-tetrahydrocannabinol (THC) in vitro on the production of testosterone (T) by the decapsulated testes of adult (2- to 3-month-old) and immature (34- to 37-day-old) mice. The results represent mean (± S.E.) concentration of T in the incubation medium at the end of a 4-hour incubation. The size of each treatment group is shown in parentheses. Abbreviation: N.S., not significant.

Concentration of THC (μg/ml)	Age of mice	Concentration of T (ng/ml)		Inhibition (%)	P
		Controls	Treated		
0.25	Adult	517 ± 58 (8)*	386 ± 27 (8)	25	< .05
2.5	Adult	517 ± 58 (8)*	426 ± 37 (8)	18	N.S.
12.5	Adult	225 ± 24 (11)	159 ± 10 (11)	29	< .02
25	Adult	517 ± 58 (8)*	71 ± 16 (8)	86	< .001
25	Immature	253 ± 33 (12)	118 ± 16 (12)	53	< .001

*Listing identical control values more than once represents comparison of several treatment groups to one control group, all run in a single incubation.

addition of THC in a 20-μl volume of ethanol, to the incubation medium, in order to achieve THC concentrations of 0.25, 2.5, 12.5, or 25 μg/ml resulted in 25, 18, 29, or 86 percent inhibition, respectively, in the accumulation of T in the incubation medium. In another experiment, with testes obtained from immature mice, the dose of 25 μg THC per milliliter resulted in a 53 percent decrease ($P < .001$) in the accumulation of T (Table 1). In a subsequent experiment with adult animals, the addition of 25 or 250 μg CBN per milliliter of incubation medium significantly ($P < .001$) inhibited the production of T (Table 2). These results complement the findings of Jacubovic and McGeer (*11*), who demonstrated that THC, CBN, and other cannabinoids can inhibit the synthesis of nucleic acids, proteins, and lipids in testicular slices in vitro.

Recently, THC has been shown to possess estrogenic activity (*15*), and some investigators have suggested that estrogens may inhibit testosterone synthesis by a direct action on the testis (*16*). However, in this incubation system, the addition of estradiol, at doses as high as 5 μg/ml, did not affect T accumulation in the incubation medium (*17*).

It is always difficult to extrapolate from experiments conducted in vitro to conditions in vivo. Although THC can concentrate in testicular tissue (*18*), the actual in vivo concentrations in human marihuana users, or in experimental animals treated with cannabinoids, may vary considerably from those used in this study. However, the results do indicate that (i) THC, in a wide range of doses (including the relatively low level of 0.25 μg/ml), can significantly reduce T biosynthesis in the decapsulated mouse testis and (ii) CBN can have a similar effect.

The ability of either cannabis or THC to lower plasma T levels in vivo has been described (*1*, *2*). We have observed that subcutaneously injecting male mice with 100 μg CBN per day for 4 days significantly reduced T levels in the plasma sampled approximately 5 hours after the last injection (1.73 ± 0.75 versus 8.50 ± 2.26 ng/ml; $P < .02$) (*19*). The similarity of the response of mouse testes in vitro to both THC and CBN suggests that nonpsychoactive constituents of cannabis can contribute to its effects on the endocrine system. Furthermore, suppression of testicular function by CBN, or other nonpsychoactive components of marihuana, could account for some of its effects on androgen-dependent behaviors.

The mechanism of action of marihuana on testicular function remains to be elucidated, but our results indicate that the reduction in peripheral T levels observed in vivo (*1*, *2*) impaired spermatogenesis (*3*, *4*), and decreases in androgen-dependent behaviors (*6*, *7*) may be due, at least in part, to a direct inhibitory effect of cannabinoids on the production of T by the testis.

Table 2. The effect of treatment with cannabinol (CBN) in vitro on the production of testosterone (T) by the decapsulated testes from adult mice. The results represent mean (± S.E.) concentration of T in the incubation medium at the end of a 4-hour incubation.

CBN concentration (μg/ml)	T ng/ml	N	T Inhibition (%)	P
	368 ± 27	9		
25	99 ± 10	9	73	< .001
250	18 ± 5	8	95	< .001

S. Dalterio
A. Bartke, S. Burstein
Worcester Foundation for Experimental Biology, Shrewsbury, Massachusetts 01545

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Effects of Marihuana Use on Body Weight and Caloric Intake in Humans

ISAAC GREENBERG*, JOHN KUEHNLE, JACK H. MENDELSON, and JERROLD G. BERNSTEIN

Alcohol and Drug Abuse Research Center Harvard Medical School, McLean Hospital, Belmont, Massachusetts, U.S.A.

Abstract. Body weight and caloric intake were measured in a group of heavy and casual marihuana users prior to, during and following 21 days of marihuana smoking under research ward conditions. A group of control subjects were studied under identical conditions, but they did not smoke marihuana. Both heavy and casual marihuana users had a significant increase in caloric intake and gained weight during the marihuana smoking period. Heavy and casual users gained an average of 3.7 and 2.8 lbs respectively during the first 5 days of marihuana smoking. In contrast, control subjects gained only a small amount of weight (0.2 lbs) during the same time interval. Water retention did not appear to be a major factor in weight gain by the marihuana users. These findings are in agreement with both anecdotal reports and previous experimental data that marihuana use is associated with increased caloric intake and weight gain.

Key words: Marihuana smoking – Weight gain – Experimental setting – Caloric intake.

Marihuana is commonly believed to enhance food intake in man. Anecdotal accounts of increased food ingestion associated with marihuana smoking (Siler et al., 1933; Haines and Green, 1970; Snyder, 1971) have only recently been assessed in clinical studies (Hollister, 1971; Williams et al., 1946). Hollister (1971) found that subjects ingested more of a chocolate milkshake preparation after 0.5 mg/kg oral delta-9 THC than after placebo. When offered the milkshake 3 h post-drug, marihuana subjects consumed 731 ml vs. 503 ml ingested by the placebo group. Chronic exposure to marihuana (39 days) or pyrahexyl, a THC analogue, (28 days) was also associated with weight gain (Williams et al., 1946).

In a recent study, Regelson et al. (1974) administered delta-9 THC to patients with cancer to determine if the drug would retard chronic weight loss. In a preliminary communication, these investigators report the delta-9 THC appeared to stimulate appetite and the patients gained weight. However, no data concerning amount of weight gained or calories ingested was reported.

The present study was part of a larger group of experiments designed to assess the effects of chronic marihuana use on various biological and behavioral functions (Mendelson et al., 1974). This report focuses upon the influence of marihuana smoking on food intake and body weight.

METHODS

Subjects. Male volunteers were recruited through advertisements placed in local newspapers. Psychiatric and medical examinations were carried out, and only those subjects in good physical and mental health were selected for participation in the study. Twelve 'casual' and fifteen 'heavy' marihuana users were studied compared with ten subjects who served as controls.

Casual users reported a mean duration of 5.3 years marihuana use with a monthly smoking frequency of 11.5 times. Heavy users reported a mean duration of marihuana use of 5.6 years and a monthly smoking frequency of 42 times. Both groups were matched as closely as possible with regard to socioeconomic background, intelligence and level of education. Further background information about the subjects is presented in Table 1.

Ten control subjects were exposed to identical ward conditions. These subjects had a past history of casual alcohol use and could work for money or alcohol on the research ward. Control subjects did not have access to marihuana or other drugs. As Table 1 indicates, the backgrounds of the control subjects were comparable to the casual marihuana users in all relevant respects. During the study they drank virtually no alcohol (average 1/5 oz. per day) and therefore qualify as drug-free controls.

Marihuana. All marihuana smoking had to be done at time of cigarette purchase, under the observation of a staff member.

* *Send offprint requests to:* Isaac Greenberg, Ph.D., Alcohol and Drug Abuse Research Center, McLean Hospital, 115 Mill Street, Belmont, Mass. 02178, U.S.A.

Table 1. Background characteristics and previous drug-taking experience: casual and heavy marihuana smokers

	Casual users ($N = 12$)		Heavy users ($N = 15$)		Controls ($N = 10$)	
	Mean	(SD)	Mean	(SD)	Mean	(SD)
Age	23.3	(1.1)	23	(1.6)	23	(1.5)
Years formal education	14.5	(1.4)	13.6	(1.5)	15.1	(1.6)
Years used marihuana	5.3	(1.1)	5.6	(1.9)	6.4	(2.3)
Marihuana use (times/mo)	13.0	(6.2)	41.0	(26.4)	3.4	(1.3)
Alcohol use (times/mo)	9.3	(8.0)	19.9	(10.0)	6.9	(4.1)

A detailed report of the experimental analysis of marihuana acquisition and use has been presented elsewhere (Mendelson et al., 1972). Unused portions of smoked marihuana cigarettes were returned to the staff to insure that 'roaches' were not accumulated and smoked without staff knowledge. Since studies were carried out on an inpatient hospital research ward, staff were able to insure that subjects did not use drugs other than marihuana.

Cigarettes containing approximately 1 g of marihuana were obtained from the National Institute of Mental Health (NIMH) in lot standard dosage form. Each cigarette contained approximately 1.8–2.3% THC as assayed by the NIMH. Actual content analysis of the marihuana using ethanol-Soxhlet and Modified Lerner extraction procedures was as follows: cannabidiol, 0.18% ± 0.04%, Δ^8THC, 0.002, Δ^9THC, 2.06% ± 0.08%, cannabinol, 0.08% ± 0.012%.

General Design. The investigation was carried out on a four-bed clinical research ward of the Alcohol and Drug Abuse Research Center at the McLean Hospital. Each study consisted of three consecutive phases: (1) a pre-drug 5-day baseline, (2) a 21-day period during which marihuana (or alcohol for control subjects) was available, and (3) a post-drug period of 5 days duration. All other conditions were identical for the marihuana and for the alcohol control subjects.

Food was prepared in the cafeteria of McLean Hospital and was brought to the research ward and served by nurses or mental health workers. The type and amount of food eaten was recorded and caloric intake calculated. Subjects were also permitted to choose their favorite snack foods and both the cafeteria and snack foods were supplied free to the subjects. Body weight was recorded each morning at 8:00 a.m. Urine samples were collected on a 24-h basis for all the casual and 11 of the 15 heavy marihuana users.

RESULTS

Daily body weight and caloric intake are reported for the heavy and casual users and the control group. Changes in body weight and caloric intake during successive 5-day periods of the study were analyzed with paired t-tests. Comparisons were made between the pre-drug control period and the first 5 drug days (study days 6–10) and also between the last five drug days (study days 22–26) and the post-drug phase. Body weights were obtained at 8:00 a.m. and represent food consumption during the previous day. Thus, post-drug body weights are plotted for a 4-day (days 28–31) rather than a 5-day (days 27–31) period in Figure 1.

Heavy marihuana users showed a significant ($P < 0.01$) increase in caloric intake and body weight following initiation of drug use (Fig. 1). Although body weight continued to increase during the drug phase, caloric intake decreased, but remained above baseline pre-drug levels. Upon termination of the smoking phase of day 26, both body weight and caloric intake decreased significantly ($P < 0.01$). The number of marihuana cigarettes smoked per day, displayed across the top of Figure 1, progressively increased during the 21-day drug phase; there was no clear relationship, however, between the number of marihuana cigarettes smoked by any single subject and the amount of food consumed. In fact, as Figure 1 indicates, the highest weight gains during the first five drug days corresponded to the least amount of marihuana use (4.29 cigarettes per day).

The casual user group (Fig. 2) also demonstrated increases in both body weight and caloric intake. Both measures increased significantly during drug availability and use ($P < 0.05$) and caloric intake decreased significantly following cessation of marihuana use ($P < 0.01$). However, body weight loss following cessation of marihuana use did not reach a statistically significant level. As with the heavy user group, no clear dose-weight relationship emerged for any subject. Once more, the high initial increases in body weight corresponded with relatively low levels of drug use (2.02 cigarettes per day).

Control subjects (Fig. 3) sustained monotonic increases in both body weight and caloric intake during the 30-day study. This pattern is in sharp contrast to the curvilinear changes seen in both marihuana groups. Further, the magnitude of weight and caloric intake changes in the control subjects was well below that seen in the marihuana groups. Weight gain comparisons between either marihuana group and the control group were statistically significant. (Casual users vs. control: $t = 4.13$, $P < 0.005$; heavy users vs. control: $t = 4.09$, $P < 0.005$.) The control sub-

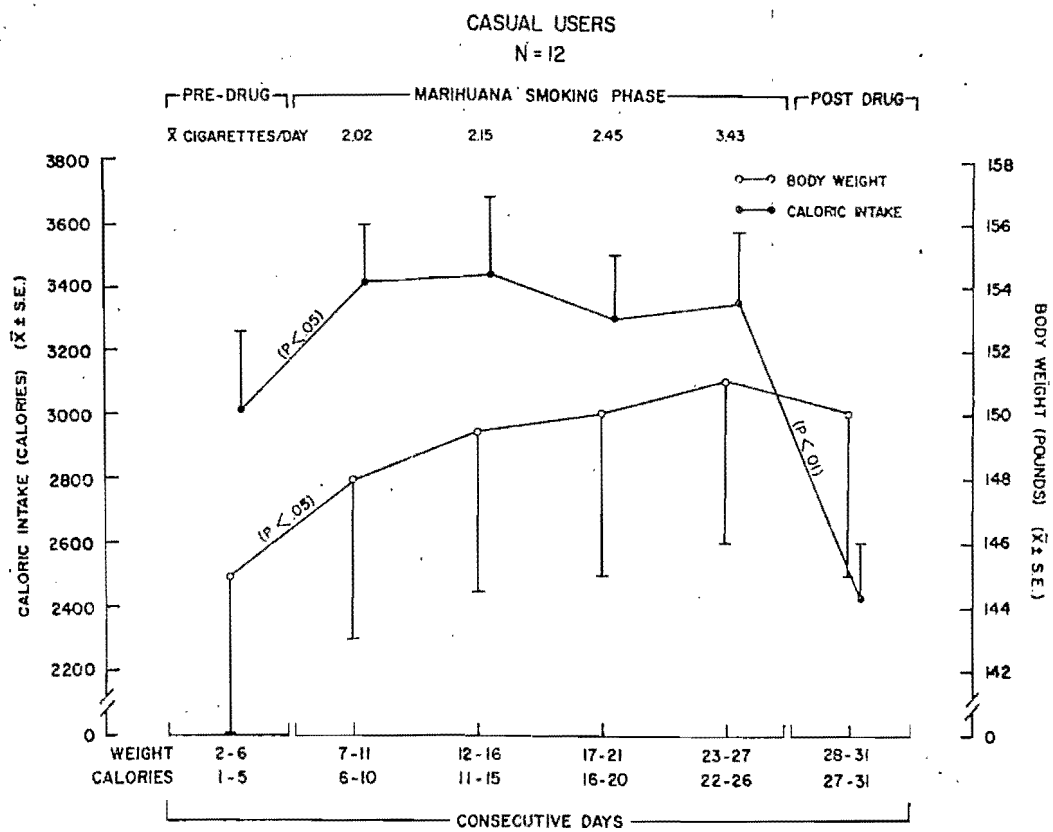

Fig. 1. Casual users (N = 12) patterns of body weight (O——O) and caloric intake (● — ●) are shown for consecutive 5-day blocks (see text). All points are group means ± standard error of the mean. At top of figure, the mean daily number of marihuana cigarettes smoked is listed for each 5-day period

jects continued to ingest food in increasingly greater amounts during the last five days of the study, while both marihuana groups had significantly depressed food ingestion levels during this period of time.

To determine if fluctuations in body-weight might be due to water retention, urine volume output was plotted as a function of time and drug phase (Fig. 4). If water retention were a function of drug use, urine volume output should have decreased upon initiation of marihuana use and should have increased with cessation of marihuana use. However, the opposite phenomena was found in the twelve casual and eleven heavy users, indicating that increased fluid intake paralleled increased food intake.

DISCUSSION

Results obtained in this study are in agreement with the findings of others on acute (Hollister, 1971) and chronic (Williams et al., 1946) effects of marihuana use on food ingestion. Hollister (1971) found that increased caloric consumption associated with acute delta-9 THC administration could be measured 3 h following drug administration. Williams et al. (1946) found that an increase in body weight occurred during a 39 day period of marihuana use. Caloric intake, however, only increased in a transient manner and then fell steadily to below pre-drug baseline levels. Evaluation of these data is difficult since the type, content and potency of the marihuana preparation smoked is not specified. Moreover, control groups were not studied to determine if non-drug related variables such as experimental setting, prison routine, type of food available, eating schedules, etc., had any influence on patterns of food ingestion. In the present study, high caloric intake was recorded throughout the smoking period for casual users, but showed a trend toward a sustained decrease below initial values for the heavy users. Since marihuana was available in our study for 21 days (vs. 39 days as described by Williams et al., 1946), it is possible that a longer period of marihuana availability would produce an initial increase followed by a depression of caloric intake.

A possible reason for a relative decrease in caloric intake after a significant initial increase at the onset of marihuana smoking may be related to gradual

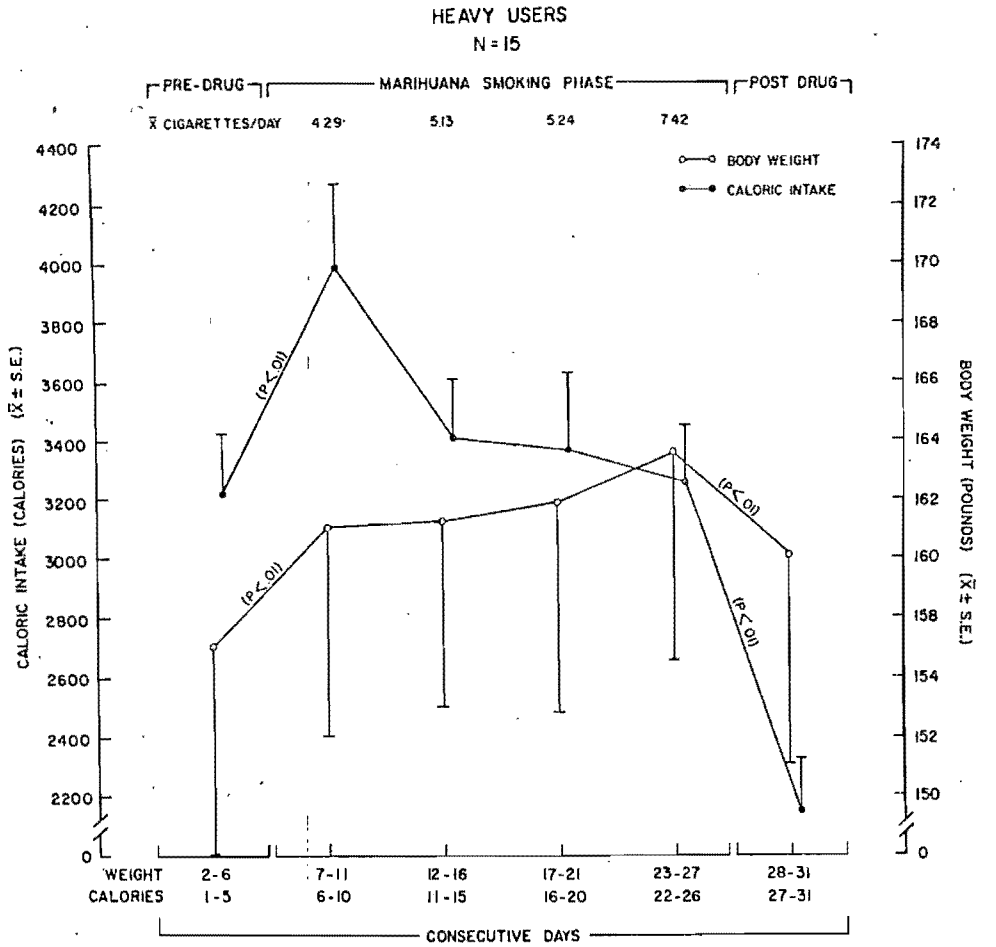

Fig. 2. Heavy users ($N = 15$) patterns of body weight (O——O) and caloric intake (● – –●) are shown for consecutive 5-day blo (see text). All points are group means ± standard error of the mean. At top of the figure, the mean daily number of marihuana cigaret smoked is listed for each 5-day period

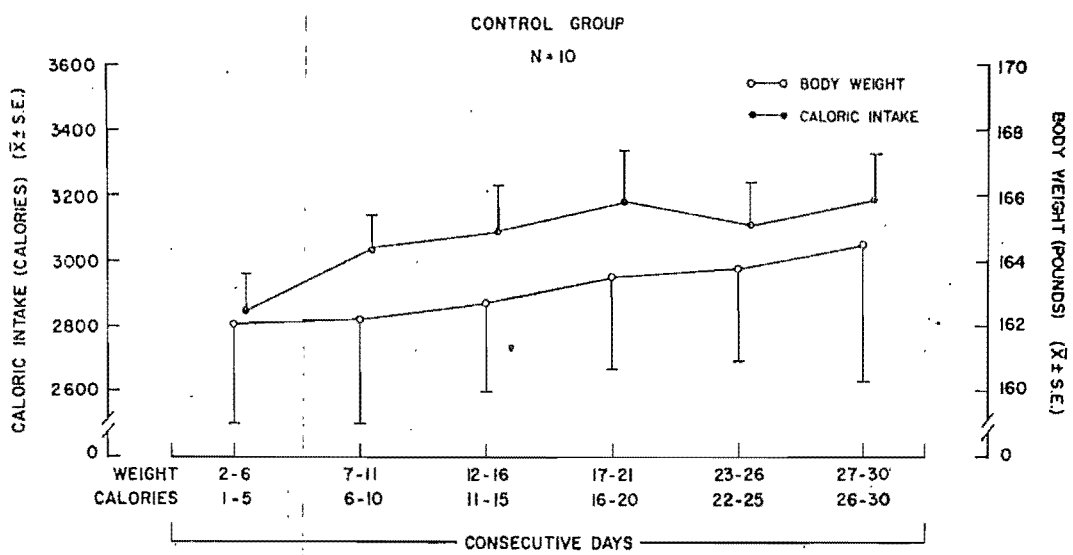

Fig. 3. Non-smoking controls ($N = 10$) patterns of body weight (O– – O) and caloric intake (●——●) are shown for consecutive 5-day blo (see text). All points are group means ± standard error of the mean

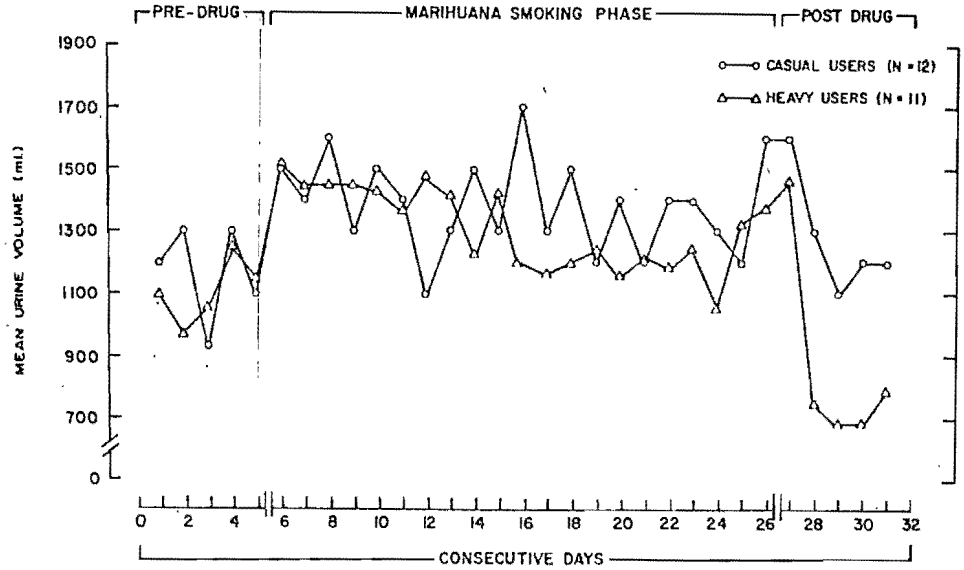

Fig. 4. Heavy (Δ——Δ) (N = 10) and casual (O——O) (N = 12) user urine volume output as a function of experimental phase

development of marihuana tolerance. It is also possible that the initial increase in food intake at the beginning of the marihuana smoking phase may have generated aversive consequences (e.g., fear of being overweight) and induced subjects to reduce food intake during subsequent marihuana smoking. In fact, subjects often verbalized their concern about gaining too much weight, but when overt dieting was reported, it began during the 5-day post-smoking period.

Control subjects gained very little weight as the study progressed. Increases averaged just over two pounds during 30 days and showed a linear trend. This phenomena might be expected considering restricted ward environment and the availability of free food.

Although there was no clear evidence that marihuana use resulted in marked fluid retention, this possiblity cannot be entirely ruled out. Benowitz and Jones (1975) have recently reported that weight gain in subjects administered daily Δ^9 THC may have been due to fluid retention and plasma volume expansion. Caloric intake was not presented in their report. The subjects in the present study showed clear changes in caloric consumption accounting for at least part of the significant weight changes. More detailed studies of total body water content are now being conducted to determine how caloric intake and changes in body water influence the weight of marihuana users.

Following administration of either pyrahexyl or delta-9 THC, rats show a decrease in food intake and in body weight (Abel and Schiff, 1969; Manning et al., 1971; Sjoden et al., 1973; Sofia and Barry, 1974). Why marihuana administration depresses food intake in laboratory animals but elevates caloric intake in humans remains unknown. Dosage factors may be as important as species differences. Human subjects control the amount of marihuana they smoke, while animals are usually given dosages proportionately many times greater than those used by humans (Elsmore and Fletcher, 1972). In the single report of THC- or marihuana-related weight gain in animals, rats were first adapted to a deprivation schedula for 150 days and then given delta-9 THC (Gluck and Ferraro, 1974). Under these conditions, rats consumed food during their daily 1 h access period in contrast to non-drug conditions. Thus, long-term adaptation to limited food access may be a necessary prerequisite for marihuana-related enhanced food intake in animals. Humans are under no such deprivation schedule, and the seemingly contradictory results between humans and laboratory animals may due be to species differences or to variables which, to date, have not been identified.

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Inhibition of a Lymphocyte Membrane Enzyme by Δ^9-Tetrahydrocannabinol in vitro

Abstract. *Delta-9-tetrahydrocannabinol (Δ^9-THC) inhibited the activity of lysolecithin acyl transferase, a membrane-bound lymphocyte enzyme, at concentrations above 1.3 μM. Stimulation of acyl transferase activity by concanavalin A, an early response in lymphocyte activation, was entirely abolished in the presence of Δ^9-THC.*

Controversy exists as to the effect of Δ^9-tetrahydrocannabinol (Δ^9-THC) on the immune system. It has been reported that lymphocyte activation in vitro can be inhibited by Δ^9-THC concentrations as low as 1.6 μM (*1*) although this has been disputed (*2*). The lipophilic nature of Δ^9-THC suggests that any such action would be mediated at the level of the lymphocyte plasma membrane, and it has been shown that the early acceleration of phospholipid turnover during lymphocyte transformation can be blocked by Δ^9-THC (*3*). Other workers have suggested that later intracellular events in the transformation process, such as DNA synthesis, are the site of Δ^9-THC inhibition (*4*). It is clear from other studies (*5*) that perturbations of membrane structure by lipophilic substances may have a profound influence on intracellular events in lymphocyte transformation. We now describe the inhibition by low levels of Δ^9-THC of a membrane-bound lymphocyte enzyme that normally participates in the events of transformation. Lysolecithin acyl transferase (E.C. 2.3.1.23) catalyzes the formation of lecithin from lysolecithin and coenzyme A–activated fatty acids. As such, the enzyme has an important role in maintaining or altering membrane structure. Its level in T (thymus-dependent) lymphocytes is rapidly increased by mitogens such as concanavalin A (Con A) (*6*). We report here the complete

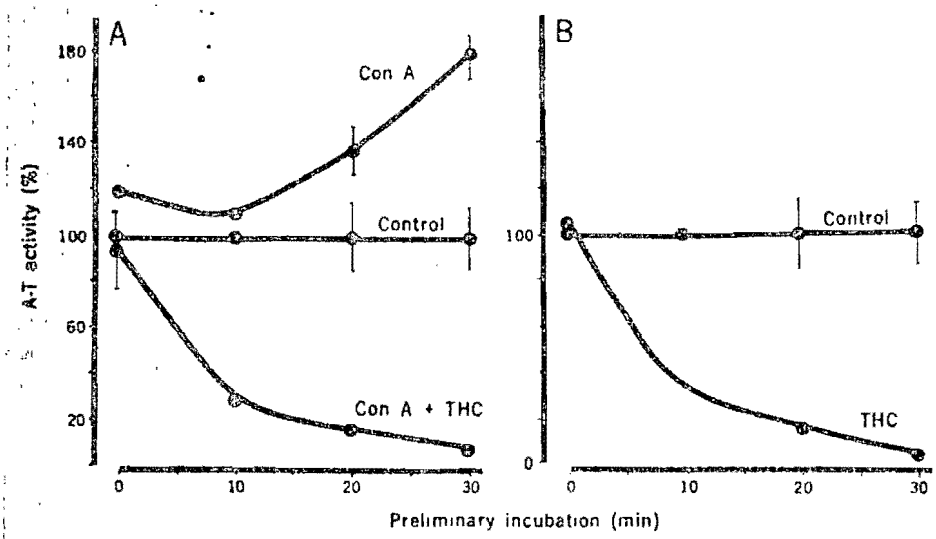

Fig. 1. Effect of Δ^9-THC on acyl transferase activity in the presence (A) and absence (B) of Con A. One milliliter of RPMI 1640 containing 10^7 lymphocytes was incubated at 37°C with 10 μg of Con A, 10 μM Δ^9-THC in 50 μl of dimethyl sulfoxide or Con A and Δ^9-THC for various times as described in the text. Controls were first incubated with 50 μl of dimethyl sulfoxide only. Acyl transferase activity was assayed by incubating the cells for 10 minutes at 37°C with 25 nmole of oleoyl-CoA and 15 to 30 nmole of [^{32}P]lysolecithin after the preliminary incubation period. The amount of ^{32}P radioactivity incorporated into lecithin in controls represents 100 percent activity. All assays were performed in duplicate, and the standard deviation is shown. The differences were significant ($P < .025$) by Student's t-test for Con A stimulation at the preliminary incubation times of 20 and 30 minutes, and for Δ^9-THC inhibition at 10, 20, and 30 minutes.

inhibition of basal and Con A–stimulated acyl transferase activity in the presence of Δ^9-THC concentrations above 1.3 μM.

Lymphocytes were prepared by gently homogenizing spleens of mice in Hanks balanced salt solution with a Teflon-glass homogenizer. Red blood cells were removed (*7*), the resultant white cells were adjusted to 10^7 cell/ml in RPMI 1640 medium, and their viability was checked by the trypan blue exclusion test. Portions (1 ml) of cells were incubated at 37°C for 10, 20, or 30 minutes or not at all (controls) in the presence of either Con A (10 μg/ml) or 10 μM Δ^9-THC in 50 μl of dimethyl sulfoxide (or both) before the assay for enzyme activity. Similar lymphocyte preparations were incubated for 30 minutes in the presence of various concentrations of Δ^9-THC in dimethyl sulfoxide before assay. Dimethyl sulfoxide had no effect on enzyme activity at the concentrations used. Acyl transferase activity was assayed by adding 25 nmole of oleoyl–coenzyme A (oleoyl-CoA) and 15 to 30 nmole of ^{32}P-labeled lysolecithin (*8*) in 0.1 ml of H_2O directly to the lymphocyte preparation. The mixture was incubated for a further 10 minutes at 37°C. Lipids were extracted in a chloroform, methanol, water system (1 : 1 : 0.8) and separated by thin-layer chromatography (*9*). The extent of acylation was calculated by comparing radioactivity (counts per minute) detected in the lecithin band with that in the lysolecithin band. Controls contained only 50 μl of dimethyl sulfoxide and were normalized to 100 percent acyl transferase activity, with all other results being calculated relative to this value. Viability studies based on the trypan blue exclusion test indicated that no significant effect on the survival of the lymphocytes occurred during incubation.

Prior incubation for 30 minutes with Con A gave maximal stimulation of the enzyme, and significant stimulation could be detected at a 20-minute incubation (Fig. 1A). Considerable inhibition of activity by 10 μM Δ^9-THC occurred in the presence of Con A (7.6 percent activity) or in the absence of Con A (5.5 percent activity). In the absence of Con A, Δ^9-THC gave complete inhibition of activity at 1.3 μM and half-maximal inhibition (K_i) at 0.35 μM (Fig. 2). No inhibition could be detected below 0.1 μM. The 2000-fold excess of substrate concentration over the Δ^9-THC concentration makes it unlikely that the inhibition is a result of lowered substrate concentration due to interaction of Δ^9-THC and substrate.

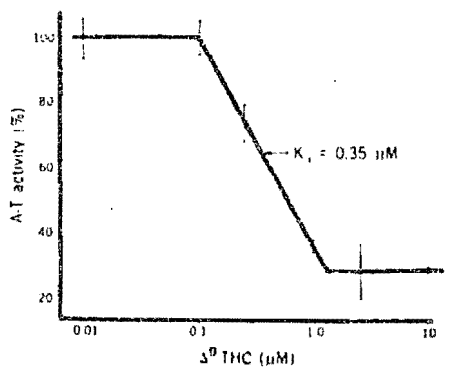

Fig. 2. Effect of Δ^9-THC on acyl transferase activity. Portions (1 ml) of RPMI 1640 containing 10^7 lymphocytes were incubated at 37°C for 30 minutes with Δ^9-THC in 50 μl of dimethyl sulfoxide. Acyl transferase activity was assayed thereafter by incubating the cells for 10 minutes at 37°C with 25 nmoles oleoyl-CoA and 15 to 30 nmoles [^{32}P]lysolecithin. The amount of ^{32}P incorporated into lecithin in controls that contained no Δ^9-THC represents 100 percent activity. All assays were run in duplicate, and the mean ± standard deviation is shown.

The earliest detected biochemical events in lymphocyte transformation are those involving lipid turnover, especially fatty acid metabolism (*10*). These changes precede the other changes, such as increased DNA and RNA synthesis by 16 hours or more (*11*). One of the causes of increased phospholipid fatty acid turnover induced in lymphocytes by mitogens such as Con A, is the increased acyl transferase activity (*6*). This increase in enzyme activity can be abolished by low concentrations of Δ^9-THC, and this effect may be responsible for an observed inhibition of fatty acid incorporation into phospholipids by 10 μM Δ^9-THC (*12*). It is of interest that substances that increase the fluidity of membranes, such as decanol, chlorpromazine, 4-chlorophenol, and barbital (*13*) will inhibit acyl transferase activity if present at much higher concentrations than that of Δ^9-THC, namely greater than $10^{-4} M$. This suggests that low concentrations of Δ^9-THC can induce changes in the lipid phase of the lymphocyte membrane that are inhibitory to the membrane-bound enzyme.

J. H. GREENBERG
M. E. SAUNDERS
A. MELLORS

Guelph-Waterloo Centre for Graduate Work in Chemistry, Department of Chemistry, University of Guelph, Guelph, Ontario, Canada N1G 2W1

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IMPAIRMENT OF ROSETTE-FORMING T LYMPHOCYTES IN CHRONIC MARIHUANA SMOKERS

SUDHIR GUPTA, M.D., MICHAEL H. GRIECO, M.D., AND PAUL CUSHMAN, JR., M.D.

Abstract Rosettes formed by circulating T and B lymphocytes obtained from 23 healthy, chronic marihuana smokers were compared with those in 23 normal control subjects who denied marihuana use. The mean percentage of T cells forming rosettes was lower in the marihuana smokers ($p < 0.005$). Nine of 23, or 39 per cent, had T-cell rosette formation lower than 2 standard deviations below the mean for control subjects. The percentages of B cell rosettes were similar in both marihuana smokers and the controls. This study suggests suppression of a T-lymphocyte subpopulation in chronic marihuana smokers. (N Engl J Med 291:874-877, 1974)

POSSIBLE immunologic effects of chronic exposure to marihuana have not received sufficient attention despite the widespread use of this drug throughout the world. Previous studies have demonstrated frequent immunoglobulin abnormalities in populations of narcotic drug abusers,[1-3] but cell-mediated immunity has not been examined closely.

Human lymphocytes are divided into two populations, thymus-dependent or T cells, which are responsible for cell-mediated immunity, and B cells which are thymus independent and responsible for antibody-mediated immunity. Human T cells form spontaneous nonimmune

From the Allergy, Clinical Immunology and Infectious Disease Division, R.A. Cooke Institute of Allergy, Medical Service, Roosevelt Hospital, and the Medical Service, St. Luke's Hospital Center, Columbia University College of Physicians and Surgeons (address reprint requests to Dr. Gupta at the R.A. Cooke Institute of Allergy, Roosevelt Hospital, 428 W. 59th St., New York, NY 10019).

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rosettes with sheep erythrocytes under appropriate experimental conditions.[4] B cells, which have surface immunoglobulin,[5] receptors for aggregated IgG[6] and complement components,[7] form rosettes with heterologous erythrocytes coated with antibody and complement. Peripheral T and B lymphocytes may be enumerated with these rosette-forming technics.

Rosette formation and other procedures, which evaluate the functions of circulating blood lymphocytes, have been instrumental in improving the understanding of lymphocyte abnormalities of a wide variety of human diseases[8-12] and may be useful in evaluation of cellular aspects of immunity associated with drug abuse.

Delta-9 tetrahydrocannabinol (THC) is reported to alter cell-mediated immune responses in rodents.[13] In a recent study of 51 young chronic marihuana smokers, a reduction was demonstrated in lymphocyte response in vitro to allogeneic cells and to phytohemagglutinin as compared to those of normal subjects.[14] Therefore, we undertook other comparisons of the circulating population of T and B lymphocytes obtained from healthy chronic marihuana smokers with that of normal subjects.

Subjects and Methods

Subjects

Twenty-three healthy marihuana users volunteered for the study. The mean age was 21.3 ± 3.5 years (± S.D.); 16 were men and seven women. Twenty-nine per cent reported use of tobacco on a daily basis, whereas 87 per cent stated that they used alcohol less than three times weekly, and none admitted to daily alcohol use. All but two denied the use of all other illicit drugs within the last three months. One reported the use of a small amount of a substance said by the supplier to be lysergic acid diethylamide (LSD) a week before the study. Of the women four were taking birth control pills, and one each reported taking multivitamins, vitamin C and chlordiazepoxide hydrochloride (Librium) within 24 hours of the study. All 23 claimed and appeared to be in good health (in Case 13 a transient upper-respiratory-tract infection developed for which she sought medical advice on the day after the initial and 10 days before the repeat study). None had evidence of self-administration of injections on physical examination.

Because some subjects had abnormal T-cell and B-cell rosette formations a repeat study was performed on 14, one to three weeks later. At the same time as the repeat lymphocyte study, a battery of intradermal tests was given with use of intermediate-strength tuberculin, 20 complement-fixing units of mumps, 100 protein nitrogen units of trichophyton, and 100 protein nitrogen units of candida. Two others had skin tests without repeat lymphocyte studies.

The controls were 23 healthy hospital employees. They were 28.8 ± 7.4 years of age and included 15 men and eight women. They denied the use of marihuana, other illicit drugs or the recent use of any medication.

Methods

Urine specimens were obtained from all but six of the marihuana users, and tested qualitatively for opiates, methadone, amphetamines, cocaine, and barbiturates. Peripheral blood was obtained for total lymphocyte counts and for chemical screening tests with use of a 12-channel AutoAnalyzer.

Ten milliliters of heparinized venous blood was drawn. Lymphocytes were isolated on Ficoll-Hypaque medium by centrifugation at 400 × g for 40 minutes. The interface ring contained mononuclear cells (approximately 90 to 95 per cent lymphocytes and 5 to 10 per cent monocytes). These cells were washed three times in Hanks's balanced salt solution. Almost 100 per cent of cells were viable as tested by trypan blue exclusion (only viable cells form rosettes). The lymphocyte were adjusted in medium Tc 199 to a concentration of 1×10^7 cells per milliliter.

T-cell rosettes. T cells were assayed by the "active" rosette test.[15] Fifty-microliter aliquots of lymphocyte suspension (1×10^7 cells per milliliter) were added to three plastic tubes containing 50 μl of gamma globulin-free calf serum (heat-inactivated and absorbed against sheep erythrocytes). This mixture was incubated at 37°C for one hour. After incubation, 50-μl aliquots of sheep erythrocytes (concentration 4×10^7 cells per milliliter) were added to each of the three tubes and spun at 200 × g for five minutes at room temperature. The pellet was gently suspended. Two hundred lymphocytes from each tube were counted under phase microscopy with magnification of 100 times, and the percentage of rosette-forming lymphocytes was calculated. A positive rosette was defined as a lymphocyte surrounded by three or more sheep erythrocytes.

B-cell rosettes. B cells were assayed for their C_3 receptors by modification of the rosette-forming technic of Bianco et al.[16] One-hundred microliter aliquots of lymphocyte suspension (concentration 2×10^6 cells per milliliter) were added to three plastic tubes containing 100 μl of 0.5 per cent trypsinized sheep erythrocytes coated with antisheep hemolysin and fresh mouse serum.

The mixture was centrifuged for five minutes at 200 × g, followed by incubation at 37°C for 30 minutes. The pellet was gently suspended and lymphocytes were counted as in the T-cell study.

Results

The percentages of cells forming rosettes under the conditions of this study are summarized in Table 1. The experimental subjects reported using marihuana for at least 12 months and averaged a duration of 4.0 ± 2.4 (mean ± S.D.) years. The frequency per week averaged 3.5 ± 1.9 and varied from one to seven times. The mean percentage of T cells forming rosettes in the 23 marihuana smokers was significantly lower than that of the 23 normal controls ($p < 0.005$). Since the normal subjects had values ranging from 20.3 to 32.8 per cent and a relatively high standard deviation, 2 standard deviations above and below the mean of the normal (i.e., 19.0 to 34.2 per cent) were used as the range of normal. With use of this criterion, there were nine of 23, or 39 per cent, with decreased numbers of T cells as measured by the "active" rosette-forming test. Six who had low values initially had low percentages in a repeat study. Of eight with normal values initially, seven were similar on repeat study, whereas a single patient had a borderline initial value of 18.5 and 16.5 per cent on repeat.

Table 1. Enumeration of T and B Lymphocytes in 23 Controls and 23 Marihuana Smokers.

Group	T Lymphocytes (%)*	B Lymphocytes (%)†
Control	26.6±3.8	11.6±2.0
Smokers‡	21.4±7.0	11.6±4.7
F-distribution significance	<0.005	NS§
Student's t-test significance	p<0.005	NS

* Mean ± SD by "active" rosette test.
† Mean ± SD (complement receptor).
‡ Absolute lymphocyte count 2159 ± 1037.
§ Not significant.

The B-cell studies showed the 23 marihuana smokers to have on the average normal percentages of B cells (C_3 receptor) forming rosettes. However, five subjects had a low

percentage of B-cell rosettes, and three were above the 7.2 to 16 per cent range of normal (2 standard deviations above and below the mean). The repeat B-cell rosette study indicated good agreement with the initial study.

The results of skin-testing of smokers showed that mumps was the most common antigen eliciting a positive response, being positive in eight of 16. Five of the 16 had no positive skin-test reactions to any of the antigens used. Three of the eight with low percentages of T-cell rosettes were nonreactive, as were two of the eight with normal percentages of T-cell rosettes.

Routine chemical screening tests by 12-channel AutoAnalyzer gave normal results in all marihuana users and controls, except for two of the former, who had glutamic oxalacetic transaminase levels of 52 and 53 mIU per milliliter. None of the marihuana users had dangerous drugs detected in the urine.

Discussion

Marihuana contains a number of possibly immunoreactive molecules, including carotenoids, chlorophylls, xanthophylls, eugenol, guaiacol, piperidine and several molecules in the cannabinoid series.[17] The possible role in allergic sensitization of the cannabinoids and probably THC has been implicated by Liskow and Parker[17] in a patient with nasal and pharyngeal pruritus, lacrimation, nasal congestion, dyspnea and wheezing immediately after smoking a marihuana cigarette. Scratch testing and passive-transfer studies confirmed an immunologic basis for the response.

Other immunologic changes in marihuana users were recently reported by Nahas et al. who found evidence of inhibition of cell-mediated immunity in THC-treated rodents[13] and marihuana smokers.[14] In the latter study the incorporation rates of ^{3}H thymidine by lymphocytes were 15,679 ± 499 (± S.E.M.) counts per minute in response to allogeneic cells in 34 and 13,799 ± 169 with phytohemagglutinin in 51 chronic marihuana smokers. This value contrasted with 26,400 ± 200 and 23,250 ± 210 in 81 unmatched, older controls. In a separate study in vitro inhibition of phytohemagglutinin-induced blastogenesis of normal human lymphocytes was observed with 1.6 to 20 μM THC.[18]

Our data suggest an association between chronic marihuana smoking and decreased numbers of a subpopulation of T cells forming "active" rosettes with sheep erythrocytes. Abnormally low percentages of T-cell-forming rosettes were found in a sizable number of unselected healthy marihuana smokers. The reproducibility of the depression in the function of the T-cell rosette was evident in repeat examination in the same patient.

Although it can never be established what the exact drug exposure had been in the marihuana smokers, the failure to detect dangerous drugs in the urines of these subjects lends support to their denials of recent use of these drugs. It is possible that there was concealed use of these or some other substances causing the T-cell changes not detectable in the urinary analytical procedures. It is not absolutely certain that these volunteers actually consumed marihuana. However, they all described the nature of the raw materials, the psychic effects, the styles of use and duration of action in terms that marihuana smokers generally use. As far as can be determined, it can be assumed that the group of persons studied were marihuana users and can be considered to have been exposed to the psychically active substances currently distributed in New York City as marihuana.

Since good correlation has been reported between spontaneous sheep erythrocyte rosette formation with human T cells and other T-cell subserved functions such as mitogen responsiveness[19,20] our data suggest a disturbance in T-cell function in marihuana smokers. On the other hand, intradermal testing showed no correlation between the presence or absence of a positive reaction to one or more of the antigens used and low or normal percentages of T cells forming rosettes.

If there is a reduction in T-cell function in marihuana users, there are important clinical questions to be raised. Cell-mediated immunity is concerned with defense against viral and fungal infections and immune surveillance against tumors. Additional studies of cancer and intracellular infections in marihuana smokers in relation to T-cell function are urgently needed in view of the increasing prevalence of chronic marihuana users in this country.

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DOSE-RESPONSE RELATIONSHIPS TO CANNABIS IN HUMAN SUBJECTS

GLENN F. KIPLINGER AND JOSEPH E. MANNO*

Lilly Laboratory for Clinical Research, Marion County General Hospital and Indiana University Medical Center, Department of Toxicology and Pharmacology, Indianapolis, Indiana

Relatively few clinical studies have been reported in which the relationship between the dose of cannabis and effect has been systematically explored. The reasons for this are centered around the chemical nature of the drug and its varied mode of use. Before the mid-1960's, there was general confusion as to the identity of the pharmacologically active principal of the crude material. Since that time there is general agreement that at least the principal active constituent of cannabis is (−)Δ^9-*trans*-tetrahydrocannabinol or more commonly Δ^9-THC. This is, of course, the pyran nomenclature rather than the monoterpenoid system. Several sensitive methods of assay for cannabinols have been developed, most of these gas-liquid chromtographic procedures. In addition, synthetic Δ^9-THC has become more available so that studies with better dosage control have been possible. Thus, most of this discussion will be derived from reports of the last 4 or 5 years.

There is a body of literature which supports the concept that there is a pharmacological difference between oral ingestion and the inhalation of smoke from cannabis products. These reported differences have clouded the issue of the overall effects of the drug, but are probably most clearly understood now in terms of administered dose of active ingredients and their relative rates of absorption. Walton (8) reviewed most of the pertinent literature up through 1938 and points out the differences between the use of cannabis resin (hashish) and smoked cannabis. When writing of the experiences of hashish users, the emphasis is placed on the hallucinatory episodes and the long duration of action. On the other hand, when smoked in the form of marihuana the reports center on the euphoria, and the more subtle alterations of time and space perception. Implicit in Walton's review is the concept of dose-effect.

In 1944, the results of studies sponsored by the Mayor's Committee on Marihuana were published (5). A portion of this report deals with clinical studies on cannabis. Doses used were usually 2 and 5 ml of a cannabis extract or three to five marihuana cigarettes. An attempt was made to relate observed effects to the administered dose and in general it was found that larger doses produced more profound effects. This was particularly true on such parameters as static equilibrium, hand steadiness, and complex reaction time where small doses produced definite effects and large doses larger effects. On other test parameters such as simple reaction time and speed of tapping the smaller doses used produced only slight or negligible effects while the larger doses produced definite

* Current address, Louisiana State University School of Medicine, Shreveport, Louisiana

impairment. Pulse rate was found to be increased with all doses by either route of administration. These effects cannot, however, be defined in terms of Δ^9-THC content of the materials used.

One of the earliest systematic studies on dose-effect relationships with drugs of this class was not done with the natural products of cannabis, but with one of the Adams' compounds. This work was done during the late 1950's and early 1960's and has been recently reported in a variety of sources, the most complete of which is the Army report released in 1970 (7). Sim studied the response of a number of volunteer subjects to orally administered graded doses of the compound known as EA1476 or DMHP or the 3-(1,'2'-dimethylheptyl) analogue of $\Delta^{6a,10a}$THC. The doses used ranged from 10 to 60 μg/kg. Pulse rates in his subjects increased and his data show a rough correlation of dose and effect but with some apparent plateauing at the higher doses (40 to 60 μg/kg). Orthostatic hypotension was a prominent finding, but again was not dose-dependent and in fact seemed more pronounced at intermediate levels. Data on the effects on psychomotor tests indicated most clearly the anticipated increasing response with increasing dose. Over a range of 10 to 60 μg/kg of EA1476, there was a definitive increased impairment on the Texas Battery (number facility and flexibility of closure), the Purdue pegboard (manual dexterity) and the Stromberg Manual Dexterity Test. In all instances, the impairment in performance correlated with the anecdotal reports of behavior and mood of the subjects.

Isbell *et al.* (2) reported on studies of dose-response analysis of Δ^9-THC in man with pulse rate and subjective questionnaire data as assay parameters. In their studies, Δ^9-THC was administered both by smoking and by mouth. When smoked, the drug was loaded on the middle one-third of a tobacco cigarette in alcoholic solution and then dried. For oral administration, the alcoholic solutions were dispersed in cherry syrup. Appropriate placebos and double-blind techniques were used. Doses were 50 and 200 μg/kg by inhalation or 120 and 480 μg/kg orally. Their data indicate highly significant, positive dose-effect relationship on both pulse rate and subjective responses. They were also able to compare the potency of THC by the two routes and found remarkable agreement between the two assay parameters. With pulse rate, they found the drug to be 2.6 times as potent when smoked and with subjective response data the potency ratio was 3.0, smoking *versus* ingestion.

The doses reported in their smoking experiments were the quantities applied to the cigarettes. Subsequent reports indicate that only 50 % of the THC content is actually delivered in the smoke (1, 5). It is, therefore, likely that the doses received in the smoke by the subjects in the study of Isbell *et al.* (2) were actually 25 and 100 μg/kg. The only significance of this observation is that the THC may be actually five to six times as potent by inhalation when delivered dose rather than cigarette content is considered. It would also be of interest to know how much THC remained in the butts of the cigarettes used in these studies.

Weil *et al.* (9) conducted a study in which the effects of two doses of cannabis were compared to placebo on several parameters. The portion of their study

most relevant to this discussion concerns itself with their naive subjects, nine in number, who received all doses administered. Their chronic users were exposed only to the high dose which is reported as 2 g of cannabis containing 0.9 % of Δ^9-THC. Their low dose was 0.5 g of the same material. The placebo marihuana consisted of the outer covering of the stalks of male hemp plants. Tobacco, placebo and mint leaves were used as fillers in all cigarettes except that the high dose contained no placebo material.

All cigarettes were marked to a uniform length with an ink line and subjects were instructed to smoke to this line. Thus, although the cigarettes contained 4.5 and 18 mg of THC (low *versus* high dose), there is no way of knowing the amount of residual THC in the butt which remained.

More pertinent perhaps is the fact that the data on pulse rate do not indicate a dose-response relationship. Both high and low dose gave increases of about 16 beats per min compared to 8 for placebo. In addition, their data indicate that with the high dose the pulse rate had returned to normal by 90 min whereas with the low dose it had not.

Weil *et al.* (9) presented data that indicated that their eight chronic users given a single 2.0 g dose of cannabis had an increase in pulse rate of 33 beats per min, double that of their naive group. However, the chronic users were not studied with the same control criteria of placebo and double-blind conditions. One possible explanation may be that the naive subjects, in spite of instructions and observation, inhaled less of the putative dose than did the chronic smokers. This, however, would not explain the fact that the chronic users were less affected on psychomotor performance than the naive.

Dose-response trends were reported by Weil *et al.* (9) for both the digit symbol substitution test and for the pursuit rotor in their naive subjects.

Melges *et al.* (6) studied the effects of multiple doses of THC administered orally as a standardized cannabis extract on mental functioning. Their doses were calibrated for Δ^9-THC content and contained 0, 20, 40 or 60 mg. These were administered to eight volunteers in a double-blind randomized block design. Their test procedures were designed to evaluate recent memory function and temporal organization. Four test situations were reported: digit span, forward and backward (a straight memory task); serial subtraction by seven's which is less dependent on recent memory and more dependent on sustained attention and long-term memory; and a goal-directed serial alternation (GDSA) task which depends on retention of recent input, mental coordination and serially indexing recent memories relevant to a goal.

They found that GDSA was profoundly affected with a dose-dependent impairment in performance. This has been termed "temporal disintegration." Dose-dependent impairment in digit span was also demonstrated. On the other hand, the serial subtraction by seven's was not significantly affected, although their data indicate a tendency toward impairment with increasing dose. It is apparent from their data that impairment of recent memory is a dose-dependent phenomenon.

We have also been concerned with various aspects of dose-dependent altera-

tions in performance and in physiological parameters (3, 4). These studies were done in collaboration with Dr. Joseph Manno, currently at Auburn University and Dr. Robert B. Forney. Subjects were male volunteers between the ages of 21 and 30 years. A brief medical history revealed no evidence of disease or of gross psychiatric abnormality. All subjects were chosen from either cigarette smokers or marihuana smokers, but none were daily users of marihuana. Also rejected were those with a history of use of potent hallucinogens.

All marihuana was administered by smoking. The marihuana was of Asian origin and assayed at 3.8% Δ^9-THC content with only a trace of Δ^8-THC. A placebo was prepared by exhaustively extracting marihuana until no cannabinoids were detected by gas-liquid chromatography analysis. This placebo marihuana was also used to dilute the 3.8% material for preparation of cigarettes containing graded doses. Experiments were done which demonstrated that only 50% of the THC content of the cigarette was actually available in the smoke. Cigarettes were, therefore, prepared on the basis of "delivered dose." That is, a cigarette that contained 10 mg of THC was considered a 5-mg dose. Doses used in our experiments were 2.5 and 5.0 mg in one study and 6.25, 12.5, 25 and 50 μg/kg in another. In each experiment, a randomized block design was used with double-blind procedures; each subject received each treatment at 1-week intervals.

All subjects were familiar with the smoking process and were asked to inhale deeply, hold the smoke, and to consume the cigarette within a period of 10 min. All cigarettes weighed 0.5 g and the placebo was indistinguishable from authentic marihuana by taste, smell or burning characteristics. All were consumed down to the charred paper by use of forceps to hold the butt. Analysis of the residue indicated that less than 10% of the THC remained.

In one study, the purpose was to examine both the effects of alcohol-marihuana combinations and varying doses of marihuana as measured by THC content. The parameters measured were pulse rate, subjective effects (Cornell Medical Index), motor performance on a pursuit meter and verbal performance with delayed auditory feedback. We were able to demonstrate an additive effect with alcohol in these studies. However, our dose-response relationships were not as clear-cut on some parameters as had been anticipated. Significant dose-response curves were developed for pulse rate and for subjective sensations. Figure 1 shows the pulse rates for 12 subjects under our different test conditions. The lower curve is the dose-response curve to THC at doses of 0, 2.5 and 5 mg delivered in the smoke. The upper curve represents the same doses of THC in the same subjects, but with a blood concentration of 0.05% ethanol. Our lowest dose of THC, 2.5 mg, produced a mean increase in pulse rate of 15 beats per min above placebo level.

In contrast, our dose-response curves for motor and mental function as measured by the pursuit meter and the delayed auditory feedback (DAF) indicated very little difference between the high and low dose. This is shown in figure 2 which is a summary of the data from four different pursuit meter tests. The lower curve is the response to 0, 2.5 and 5 mg of THC while the upper curve

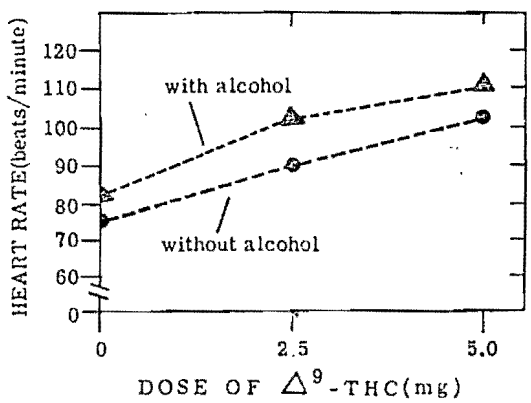

FIG. 1. Mean heart rate of 12 subjects administered doses of (—)Δ^9*trans*-tetrahydrocannabinol (Δ^9-THC) calibrated marihuana cigarettes. Lower curve, marihuana alone; upper curve, same doses in the presence of ethanol at a concentration of 0.05% in blood. (From Joseph E. Manno, Glenn F., Kiplinger, Norman Scholz and Robert B. Forney: The influence of alcohol and marihuana on motor and mental performance. Clin. Pharmacol. Ther. 12: 202–211, 1971.)

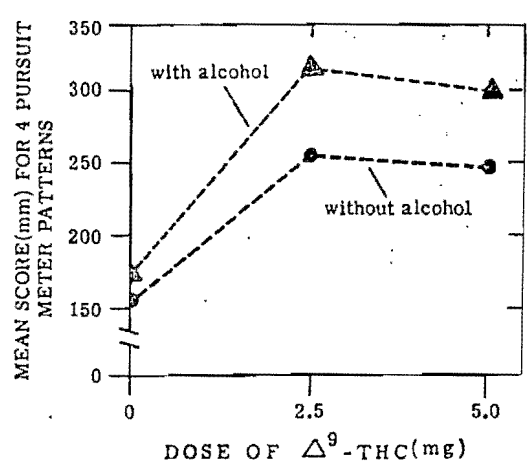

FIG. 2. Mean score for 12 subjects on four pursuit meter patterns after doses of (—)Δ^9-*trans*-tetrahydrocannabinol (Δ^9-THC) administered as calibrated marihuana cigarettes. Lower curve, marihuana alone; upper curve, same doses in the presence of ethanol at a concentration of 0.05% in blood. (See reference 4.)

is the same only in the presence of alcohol, 0.05%. Our feeling at this time was that perhaps there was some plateauing effect, or that our instruments would not allow the distinction between the two doses we were using. In order to examine this phenomenon, we decided to extend the dose response analysis to lower doses and to administer THC on a μg/kg basis. Doses chosen were 6.25, 12.5, 25 and 50 μg/kg with a placebo control. Parameters measured were essentially the same except that we added a measure of static equilibrium, and the

Addiction Research Center Inventory for marihuana effects. These results are summarized in figures 3, 4 and 5.

Figure 3 shows both the magnitude and the duration of the effect on pulse rate. If the rate at 20 min post-smoking is used as the dependent variable against dose, there is a highly significant linear dose-response curve. Notice that the duration is also dose-dependent in that larger doses have not yet returned to baseline at the termination of the experiment.

As an aside from the dose-response data, Dr. Thomas Bright working in our laboratories has shown that the increase in pulse rate can be blocked by the *beta*-adrenergic blocker, propranolol. The blocker was administered in four divided doses totaling 160 mg during the 24 hr before challenge with a cigarette calibrated to deliver 25 μ/kg of THC. Appropriate control with intravenous isoproterenol demonstrated that *beta*-blockade had been established.

Figure 4 shows the data for the pursuit meter on four different patterns. Again, there is a significant increase in error score which is linearly dependent on dose.

Figure 5 shows the effect of the same dose on static equilibrium. This is measured with an electronic device which records sway as electrical counts. Increasing counts indicate increasing sway. Stability is also measured under conditions designed to remove visual and proprioceptive cues. Thus, there are four sets of

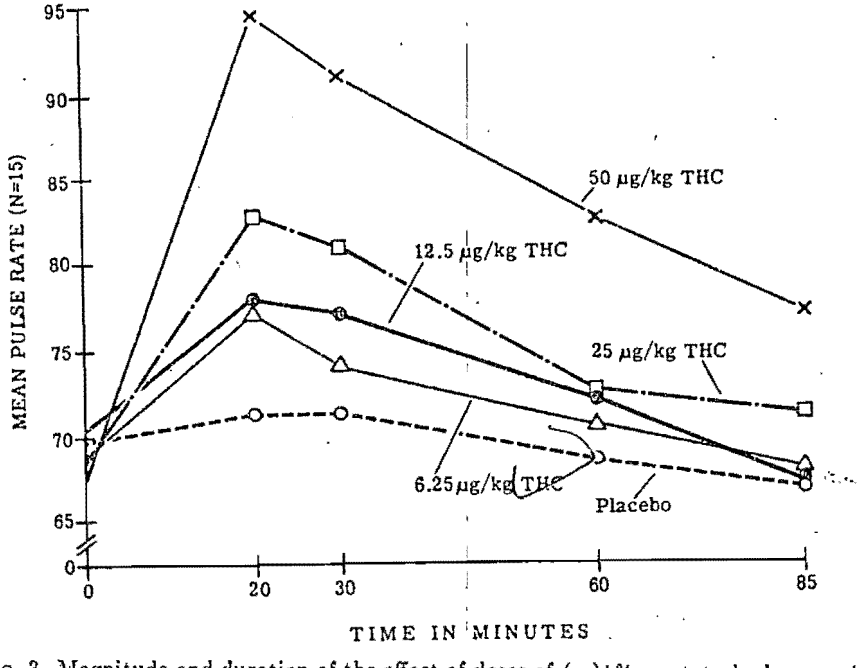

FIG. 3. Magnitude and duration of the effect of doses of (—)Δ^9*trans*-tetrahydrocannabinol (Δ^9-THC) on pulse rate. All doses administered to the same 15 subjects as calibrated marihuana cigarettes. (From Glenn F. Kiplinger, Joseph E. Manno, Bruce E. Rodda, and Robert B. Forney: Dose-response analysis of the effects of tetrahydrocannabinol in man. Clin. Pharmacol. Ther. **12**: 650–657, 1971.)

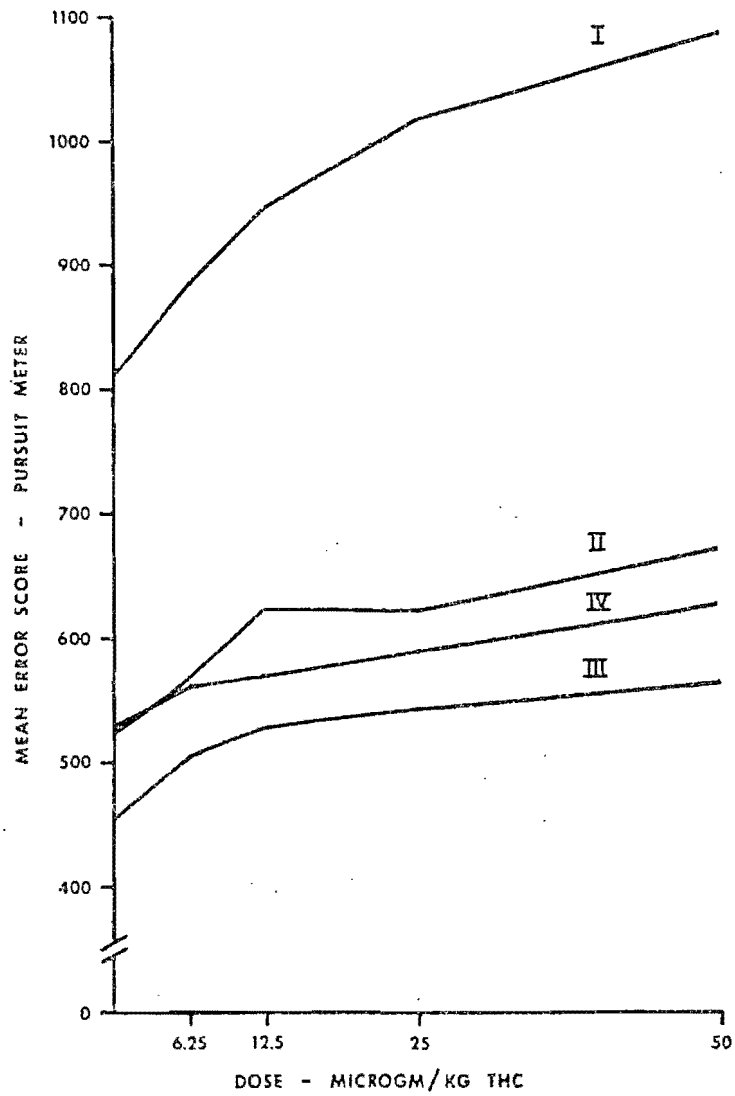

FIG. 4. Mean error score for 15 subjects on four different pursuit meter patterns. Doses of (—)Δ^9-*trans*-tetrahydrocannabinol (Δ^9-THC) were administered as calibrated marihuana cigarettes. Ordinate, error score is millimeter of deviation from a standard trace; abscissa, dose of Δ^9-THC; roman numeral refers to the four patterns used in the study. All curves show a significant dose-dependent increase. (See reference 3.)

curves: normal conditions (eye open and fixed for far vision); eyes closed; eyes open, but with a vibrator on the stand to remove or confuse proprioceptive input from the feet and legs; and eyes closed with vibrator on. All curves were parallel indicating no particular sensitivity of one condition over another.

In summary, as one might predict for an active pharmacological substance, there is a relationship between administered dose of cannabis, its constituents or

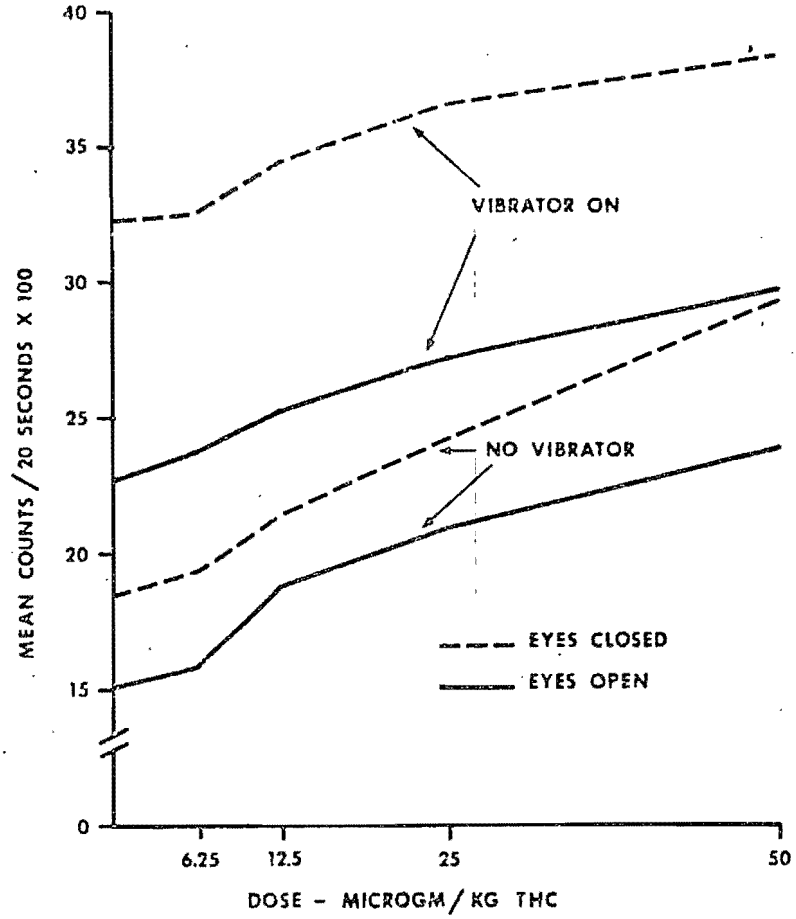

FIG. 5. Effects of (—)Δ^9-*trans*-tetrahydrocannabinol (Δ^9-THC) on static equilibrium in 15 subjects. Doses were administered as calibrated marihuana cigarettes. Ordinate, electrical counts as a measure of sway in the standing position; abscissa, dose of Δ^9-THC. See text and reference 3 for explanation of the four conditions. All curves show a significant dose-dependent increase in sway.

analogues, and the response observed. Perhaps the most consistent of these is the tachycardia characteristically produced by THC. This has been demonstrated by Isbell *et al.* (2) by both oral and inhalation routes and by us with the use of inhalation. Curiously, Weil *et al.* (9) found no such relationship in their naive subjects even though chronic users did show a pronounced tachycardia with a single dose of the same material.

Other parameters for which dose-response relationships have been demonstrated include: several psychomotor measurements (3, 4, 7); positive responses on subjective questionnaires (2, 3, 4); mental performance and short-term memory (3, 4, 6).

The hallucinatory experiences and transient psychoses produced by these derivatives are also part of the dose-response phenomenon in that they are generally observed with doses far in excess of those required for euphorigenic effects.

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Delta-9-Tetrahydrocannabinol: Localization in Body Fat

Abstract. *[^{14}C]Δ^9-Tetrahydrocannabinol (Δ^9THC) was injected subcutaneously in rats every day for 1 to 26 days. Concentrations of Δ^9THC and its metabolites, 11-hydroxytetrahydrocannabinol and 8,11-dihydroxytetrahydrocannabinol, were determined in various tissues. After a single injection, the concentration of Δ^9THC in fat was ten times greater than in any other tissue examined, and persisted in this tissue for 2 weeks. With repeated injection, Δ^9THC and its metabolites accumulated in fat and brain.*

Previous studies have shown that [^{14}C]Δ^9-tetrahydrocannabinol (Δ^9THC) persists in the plasma of man for several days after its intravenous administration (*1*) and that, after a single injection of [^{3}H]Δ^9THC to experimental animals, total radioactivity remained in fat (*2*, *3*) and brain (*4*) for several days. A major metabolite of Δ^9THC, 11-hydroxytetrahydrocannabinol (11-hydroxy THC) (*5*, *6*), is behaviorally active in animals (*5*) and humans (*7*), whereas 8,11-dihydroxytetrahydrocannabinol (8,-11-dihydroxy THC) has been demonstrated to be a nonactive metabolite (*1*, *5*, *8*).

Because of the lipophilic nature of Δ^9THC, its persistence in plasma might be due to sequestration in and slow release from fat. In chronic marihuana users the effects of Δ^9THC might result from accumulation of Δ^9THC or an active metabolite in brain. We now describe the selective accumulation and retention of Δ^9THC and its metabolites in fat after single and repeated subcutaneous doses of [^{14}C]Δ^9THC to rats.

Female Sprague-Dawley rats weighing 150 g were injected subcutaneously just below the scapula every other day with 14 μl of an ethanol solution (1 mg/ml, 17.5 μc/mg) of [^{14}C]Δ^9THC (*9*). Forty-four hours after 1, 3, 6, 9, or 13 doses of the THC solution, four rats were decapitated. The brain, lung, and parts of the liver and perirenal fat pads were homogenized, and the Δ^9THC, 11-hydroxy THC, and 8,11-dihydroxy THC were separated and measured by extraction into heptane of various polarities (*10*).

There was a tenfold greater concentration of Δ^9THC in fat than in the other tissues (Fig. 1A), and there was a fourfold increase over the initial concentration in fat with repeated injection. In brain Δ^9THC could not be detected at day 2, but by day 7 could be measured (0.37 ng per gram of tissue), and this concentration doubled by day 27.

The accumulation of 11-hydroxy THC, the active metabolite of Δ^9THC, shows a similar distribution (Fig. 1B) except that its concentration in fat, although higher than that for the other tissues, was less than that of Δ^9THC in fat. In brain, 11-hydroxy THC was undetectable at day 2 but by day 27 reached a concentration of 0.45 ng per gram of tissue.

The accumulation of 8,11-dihydroxy

THC (Fig. 1C) is similar except for fivefold greater accumulation in liver than in lung; 8,11-dihydroxy THC has been shown to be formed readily in vitro in liver but not in lung (*11*).

The retention of Δ^9THC and its metabolites in fat (Fig. 1D) and the other tissues was examined by injection of a single dose of [^{14}C]Δ^9THC and analyzing the tissues periodically over 14 days for Δ^9THC and metabolites. An approximate half-life of 5 days was found for Δ^9THC in fat, while 11-hydroxy THC and 8,11-dihydroxy THC persisted in smaller amounts over 14 days. In liver small amounts (0.44 ng per gram of tissue) of Δ^9THC and its metabolites were present for 14 days, while in lung similar amounts were present for 2 days only.

Estimates were made of the residual unidentified polar metabolites (*12*). After 13 doses of [^{14}C]Δ^9THC, there were negligible amounts in brain, small amounts in fat (0 to 5 ng per gram of tissue) and lung (3 to 10 ng per gram of tissue), and large amounts (30 to 60 ng per gram of tissue) in liver. The amounts of polar metabolites accumulating in liver and lung were greater than the sum of Δ^9THC, 11-hydroxy THC, and 8,11-dihydroxy THC in these tissues.

The disappearance curve for Δ^9THC in the plasma of man (*1*) and of total radioactivity in rats (*2*) shows an initial rapid decline (half-time of minutes) after intravenous administration followed by a long slow phase (half-time of days), suggesting that Δ^9THC is rapidly taken up in tissues or metabolized or both. Since the disappearance curve for total metabolites is also biphasic (*1*), and Δ^9THC is present in plasma for a week after a single intravenous dose (*1*), it is probable that tissue sequestration, especially in fat, plays a dominant role in the disposition of Δ^9THC. The importance of fat localization of drugs in explaining their duration of action has been shown for drugs such as thiopental (*13*), dibucaine (*14*), and DDT (*15*). These drugs show a similar biphasic disappearance curve from plasma, a high localization in fat, and a comparable rate of accumulation in fat with repeated administration. DDT reaches maximum levels in fat of man after 1 year of the normal amounts found in food (*16*). If the period of injection of Δ^9THC had been extended over a longer time, a plateau for Δ^9THC accumulation in fat might reach a much higher value than that reported in Fig. 1A. With starvation, DDT concentrations increase in rat brain because of mobilization from fat stores (*17*). It would be of interest to study this phenomenon in the chronic marihuana users who report flashback (*18*).

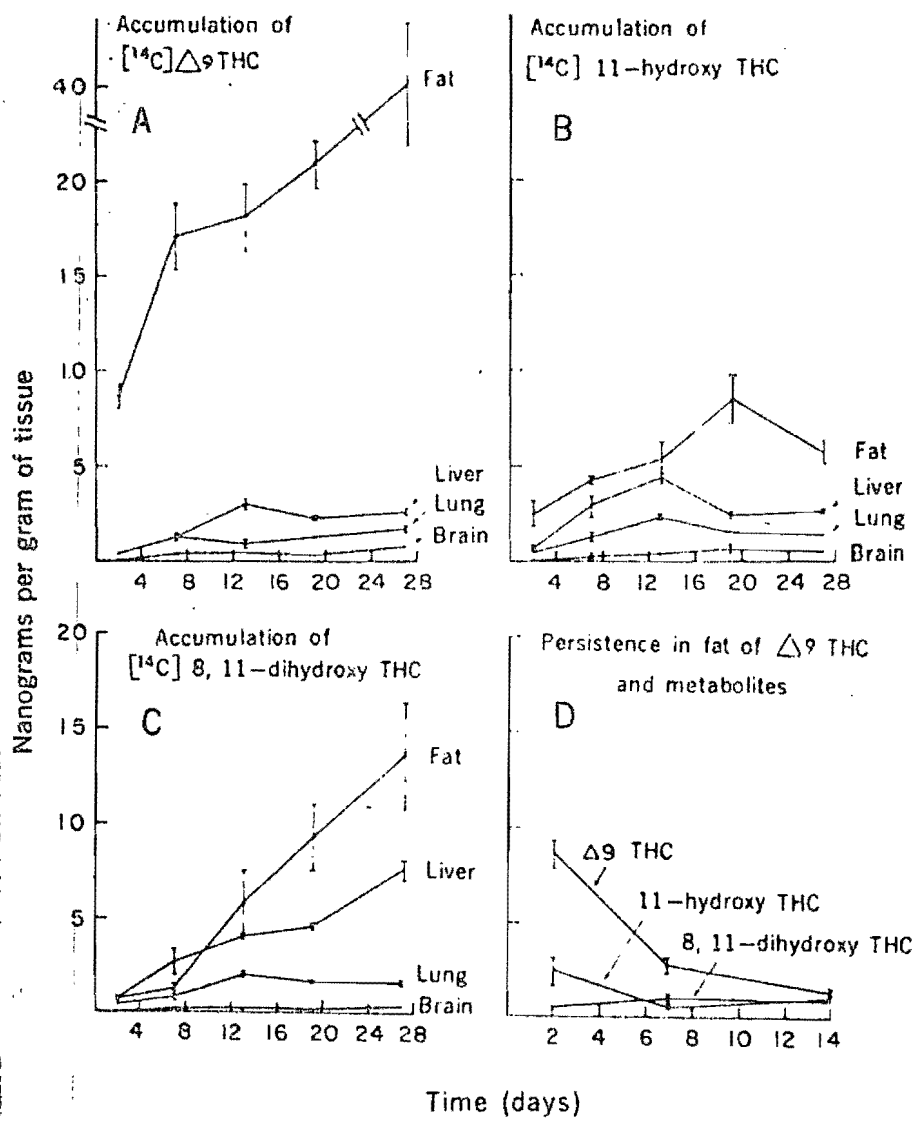

Fig. 1. The distribution of Δ^9THC, 11-hydroxy THC, and 8,11-dihydroxy THC in rat tissues after repeated subcutaneous doses of [^{14}C]Δ^9THC. (A to C) The [^{14}C]Δ^9THC was given every other day for the stated number of days. (D) A single dose of [^{14}C]Δ^9THC was given, and tissues were examined at the times indicated. Results are expressed as mean ± standard error of the mean for four animals at each time point.

DAVID S. KRA
JULIUS AXELR

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014

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9. A Hamilton microsyringe was used. E nation of the injection site after 13 inje revealed no gross pathological changes, an ethanol extract of the tissues at th revealed 500 to 1000 count/min.
10. The tissues were homogenized in three vo of KH_2PO_4–Na_2HPO_4 buffer (0.05*M*, pH with a Polytron homogenizer. Four vo of heptane were added, and the mixtur agitated with a Vortex mixer for 1 m then shaken in a mechanical shaker f minutes. After centrifugation at 1500 10 minutes, the organic extracts were with a gentle stream of nitrogen at temperature to a volume of 0 to 4 m which 1 ml of ethanol and 10 ml of pho

Phytohemagglutinin-Induced Lymphocyte Transformation in Humans Receiving Δ^9-Tetrahydrocannabinol

Abstract. *Eight otherwise healthy male chronic marijuana smokers were hospitalized for a period of 30 days. Initially they received placebo, then a sustained dose of 210 milligrams of Δ^9-tetrahydrocannabinol (Δ^9-THC) per day for 18 days, followed by placebo. Lymphocyte responses to phytohemagglutinin were examined during each of these periods. Neither the daily ingestion of marijuana extract containing 210 milligrams of Δ^9-THC for 18 days nor the history of chronic marijuana smoking had a depressive effect on the lymphocyte responses of these subjects to phytohemagglutinin.*

A controversy currently exists regarding the status of cell-mediated immunity in otherwise healthy marijuana smokers. Nahas *et al.* (*1*) reported that in vitro blastogenic responses to phytohemagglutinin (PHA) and allogeneic cells in chronic marijuana smokers were depressed by 40 percent, bringing them to levels seen in patients with cancer or uremia or transplant recipients with iatrogenic immunosuppression. Although this work has not been directly confirmed, Gupta *et al.* (*2*) found a 5 percent mean decrease in the rosette-forming capacity of lymphocytes from chronic marijuana smokers, supporting the concept of a T-cell defect. By contrast, Silverstein and Lessin (*3*) noted no difference between chronic marijuana users and normal controls as evaluated by in vivo response to skin sensitization with 2,4-dinitrochlorobenzene, a technique for determining the functional integrity of cell-mediated immunity. Most recently White and coworkers (*4*) have reported that they could find no significant difference between the blastogenic responses of lymphocytes from long-term marijuana smokers and matched control subjects in response to the mitogens PHA and pokeweed. This is in direct conflict with the earlier report by Nahas *et al.* (*1*), underscoring the need for further investigation of this subject. A recent report indicates that oral delta-9-tetrahydrocannabinol (Δ^9-THC) is an effective antiemetic in patients receiving cancer chemotherapy (*5*). The use of Δ^9-THC in these patients would be undesirable if it contributed significantly to immune suppression.

A major problem in interpretation of these studies has been the experimental variation introduced by the use of chronic marijuana smokers who smoke material of unknown potency at unspecified times in a nonregulated manner. The purpose of this study has been to compare the PHA-induced blastogenesis of normal human subjects and subjects receiving a known quantity of Δ^9-THC, the major psychoactive component of marijuana, at scheduled time intervals under carefully controlled hospital conditions.

Eight male volunteers between the ages of 21 and 30 were selected for this study. They were part of an extensive study of many marijuana effects. All were regular marijuana smokers (mean frequency of 13.5 marijuana cigarettes per week for an average duration of 3.5 years). They denied the regular use of drugs (other than alcohol and tobacco), particularly narcotics, barbiturates, and amphetamines. A complete medical history and physical examination confirmed their good mental and physical health. Subjects were asked to refrain from all drug usage for 1 week prior to admission to the Clinical Research Ward of Langley Porter Neuropsychiatric Institute in San Francisco, California. They were then admitted in groups of two and kept under constant observation for the 30 days of the hospitalization period.

Each subject received a capsule containing either placebo (0.2 ml of ethanol) or drug (a crude marijuana extract in 0.2 ml of ethanol, recently assayed for Δ^9-THC content) (*6*) every 4 hours during the entire hospitalization period. Placebo was given in a double blind situation to all subjects for the first 6 days, during which time baseline data were acquired. Then rapidly increasing doses of drug were given until a maximum of 210 mg of Δ^9-THC per day in divided 30-mg doses was reached. This dose level was maintained for 18 days, followed by an abrupt switch to placebo for the remaining 4 days of hospitalization. In addition to the oral doses, all subjects smoked a 1-g marijuana cigarette just before and on the last day of prolonged oral administration. Blood samples were drawn for lymphocyte cultures during the initial placebo period just before the drug was started, at the end of the period of prolonged drug administration, and at the end of the final placebo period. The control subjects were aged 21 to 50 years and were recruited from laboratory staff and friends. Most had never used cannabis and none had any in the previous 6 months. They all were in excellent health.

Blood samples were drawn with 10

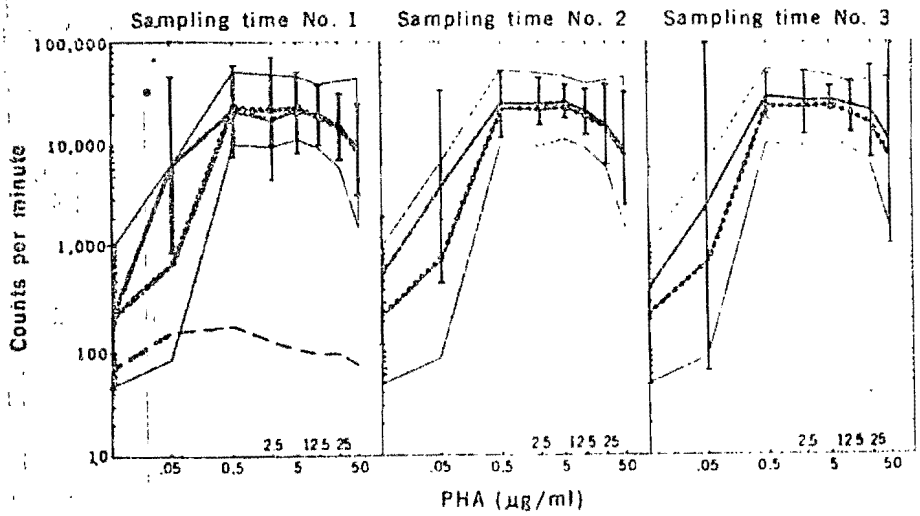

Fig. 1. The lymphocyte responses to phytohemagglutinin for eight patients on the drug protocol. Sampling time No. 1 is at the end of the initial placebo period, sampling time No. 2 is at the end of the period during which the subjects received 210 mg of Δ^9-THC per day, and sampling time No. 3 is after rapid withdrawal of the drug. In each graph the dotted line represents the mean, and the shaded area ± 2 standard deviations (S.D.), for normal control lymphocytes tested simultaneously with the subjects' lymphocytes (N = 15). The solid line is the mean for the subject group with the vertical bars representing ± 2 S.D. The dashed line in the lower part of the first graph is the PHA dose-response curve of lymphocytes from a renal transplant patient on immunosuppressive drugs.

units of preservative-free heparin per milliliter and processed according to the method of Mangi and Kantor (*7*) for short-term storage of human lymphocytes prior to in vitro stimulation. Subject and control samples drawn at the same time were coded, packed together in the same insulated cartons with no refrigerant, and shipped via air express to Ann Arbor for the lymphocyte studies. Cultures were established within 24 hours of venipuncture. Lymphocyte suspensions were prepared by separation on a Ficoll-Hypaque gradient (density 1.077) and lymphocyte incubations were established by a modification of the microculture system described by Thurman *et al.* (*8*). Each culture contained 1×10^5 lymphocytes in 0.2 ml of medium 199 (Grand Island Biological) containing penicillin, streptomycin, amphotericin B, and glutamine plus 25 percent serum, either autologous or homologous. Dose-response curves to PHA (phytohemagglutinin-P, Difco) employed triplicate cultures at seven doses ranging from 0.05 to 50.0 μg/ml. The cultures were incubated at 100 percent humidity, 37°C, 97 percent air, and 3 percent CO_2 for 66 hours, at which time 0.2 μc of [^{3}H]thymidine, specific activity 25 c/mmole (Nuclear Dynamics), was added. After an additional 6 hours of incubation the cultures were harvested with a multiple automated sample harvester and counted in a scintillation counter. Lymphocyte blastogenesis was measured by the incorporation of [^{3}H]thymidine into DNA and expressed as the average number of counts per minute per culture. Dose-response curves of the subjects and controls at each sampling time were prepared by using geometric statistics (*9*).

These results are shown in Fig. 1. The dose-response curves of six subjects for whom complete data were available for all three time intervals were compared by analysis of variance to the dose-response curves of six contemporaneous controls. The controls were selected randomly from a pool of 15 controls (*10*). There was a statistically significant ($P < .05$) higher unstimulated background count at times 2 and 3 during and shortly after high doses of Δ^9-THC. There was a statistically significant increased response to PHA at all doses during and immediately after high doses of Δ^9-THC ($P < .0005$), but this effect was abolished if the data were normalized to account for the higher background counts of the subjects. At all time periods the response of the subjects to a very low (0.05 μg/ml) dose of PHA is greater ($P < .05$) than that of normal controls. This increased response persists when the data are normalized to account for the higher background counts. The analysis clearly indicates that at very low doses of PHA the subjects had enhanced responses. The responses to PHA in total are statistically increased in the subject group, although we are unwilling to assign any biologic significance to the small differences observed. The results obtained on lymphocytes from a kidney transplant patient whose blood was coded and shipped along with the subjects' lymphocytes are included in the graph to illustrate the magnitude of suppression of lymphocyte response seen with potent immunosuppressive agents.

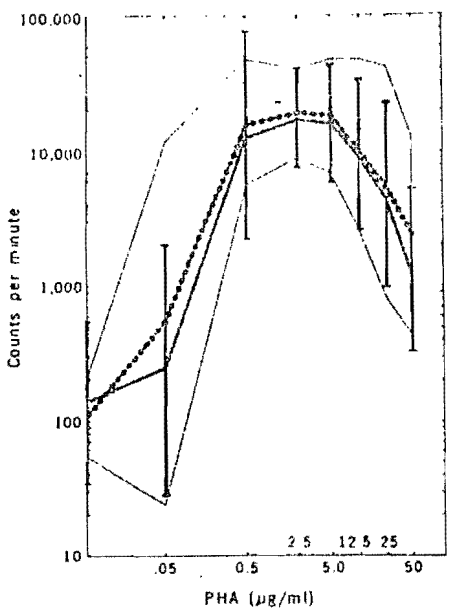

Fig. 2. Lymphocyte responses to phytohemagglutinin (PHA) in seven chronic marijuana smokers. The dotted line represents the mean and the shaded area ± 2 S.D. for normal controls in this laboratory. The solid line represents the mean and the vertical bars ± 2 S.D. for chronic marijuana smokers whose lymphocytes were studied 8 to 72 hours after smoking marijuana.

No differences in the blastogenic response were observed when the subjects' lymphocytes were incubated in homologous type AB+ serum as compared to autologous serum. Furthermore, in experiments not shown, no alteration of the response of normal lymphocytes was seen when serum from patients receiving 210 mg of Δ^9-THC per day was added to the supporting medium. We conclude from these observations that (i) these chronic marijuana smokers had evidence of an increased lymphocyte response to a suboptimal stimulating dose of PHA; (ii) no evidence of depression of lymphocyte responses to PHA could be ascribed to their prior marijuana use or to oral Δ^9-THC administration; and (iii) the serum of these subjects did not inhibit the blastogenic response to PHA of normal lymphocytes.

In the absence of a sensitive and practical assay for Δ^9-THC serum levels, and because our data are from subjects receiving oral cannabis rather than smoking marijuana, the following observations are provided to confirm that adequate drug absorption does occur via this route. The usual cardiovascular effects following a single marijuana cigarette are tachycardia and peripheral vasodilata-

tion (*11*). After continuous administration of Δ^9-THC, however, the initial tachycardia was followed in some subjects by bradycardia. In this group of subjects the mean pulse rate was 60 per minute for the first 5 days (placebo period), rose to 66 per minute on the first day of cannabis administration, and dropped to a low of 54.3 per minute after 12 days of drug administration. Other cardiovascular effects consistent with Δ^9-THC intoxication (*12*) included a fall in mean systolic blood pressure of 14 mm-Hg, a fall in mean diastolic blood pressure of 17 mm-Hg, and an associated weight gain which averaged 4.54 kg per subject during the first 16 days of drug administration. The weight gain, due to fluid retention, was lost within 48 hours of stopping drug administration. Typical marijuana effects were also noted by changes in electroencephalograms, autonomic nervous system, perceptual motor tasks, endocrine system functions, and ward behavior. The participants reported subjective feelings of intoxication during the period of drug administration. Other experiments with similar subjects have shown that Δ^9-THC given orally produces a near complete cross-tolerance to single acute doses of smoked marijuana administered at various points during the oral dosage schedule (*12*).

An additional seven chronic marijuana smokers were studied. This group consisted of males and females aged 20 to 26 years. The group averaged 4.7 marijuana cigarettes per week with a mean duration of smoking of 4.6 years. The blood samples were drawn 8 to 72 hours after the last use of marijuana. Controls consisted of non-drug-using laboratory personnel in the same age range. Control and experimental lymphocytes were cultured at the same time. As indicated in Fig. 2, the mean PHA responses of marijuana smokers fell well within the normal range. These results are in agreement with those found in the hospitalized group and with those of a similar study reported by White and co-workers (*4*).

In summary, in otherwise healthy chronic marijuana smokers, eight of whom were observed under controlled conditions and given pharmacologic amounts of Δ^9-THC, normal lymphocyte responses to PHA were observed.

R. JANE LAU, DAVID G. TUBERGEN
MASON BARR, JR.
EDWARD F. DOMINO
Departments of Pediatrics and Pharmacology, University of Michigan, Ann Arbor 48109
N. BENOWITZ, REESE T. JONES
Department of Psychiatry, University of California, San Francisco 94143

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MINIREVIEW

THE PHYSIOLOGIC DISPOSITION OF MARIHUANA IN MAN

Louis Lemberger and Alan Rubin

Lilly Laboratory for Clinical Research
Lilly Research Laboratories
and the
Departments of Pharmacology and Medicine
Indiana University School of Medicine
Indianapolis, Indiana 46202

Marihuana and hashish are the most widely used illicit drugs. They are derived from the hemp plant, Cannabis sativa. Among the diverse chemicals in the plant, more than 20 so-called cannabinoids have been isolated and their chemical structures elucidated (1). In 1965, Mechoulam and coworkers (2,3) isolated Δ^9-tetrahydrocannabinol (Δ^9-THC) from cannabis extract and demonstrated that it was responsible for the psychopharmacologic effects of cannabis in animals. Later, Isbell (4) and Hollister et al. (5) confirmed these findings in man. This paper reviews the physiologic disposition of Δ^9-THC in man; the disposition of Δ^9-THC in animals has been reviewed elsewhere (6,7,8).

Absorption

Various vehicles for the oral administration of Δ^9-THC have been studied extensively in man (9). H^3-Δ^9-THC was either dissolved in sesame oil or ethanol, or was emulsified in 5.5% sodium glycocholate (a bile salt). Volunteers who ingested these preparations reported an intense and unpleasant psychological "high" when sodium glycocholate and sesame oil were used as the vehicles for Δ^9-THC, whereas they reported a moderate and pleasant high when ethanol was used as the vehicle. The "high" reported after Δ^9-THC in sodium glycocholate appeared 15-30 minutes after administration, compared to 60 minutes when sesame oil or ethanol was the vehicle. Plasma levels of total radioactivity were considerably higher and of longer duration after the sesame oil and sodium glycocholate vehicles than after the ethanol vehicle, and appeared to parallel the psychologic high. The radioactivity in blood rose most rapidly and reached the highest levels when sodium glycocholate was the vehicle. This correlated well with the intensity of observed and reported drug effects. The radioactivity in plasma rose most slowly and achieved the lowest peak level when ethanol was the vehicle. Others have reported that 90-95% of a dose of Δ^9-THC is absorbed from the gastrointestinal tract after oral ingestion of this drug dissolved in ethanol (5, 10,11). In these studies, the plasma levels of total radioactivity increased slowly and reached a peak at the time when maximal psychologic effects were reported (3 hours).

Isbell (4) compared the effects of Δ^9-THC in man after giving the drug via the pulmonary and oral routes. It was necessary to administer about three times as much drug orally to achieve the same psychologic effects as after inhalation and he suggested that more efficient absorption via the pulmonary route might be an explanation. In support of this suggestion, Lemberger and coworkers (10,12) reported higher initial plasma levels of radioactivity after subjects smoked marihuana cigarettes containing ^{14}C-Δ^9-THC than after oral administration of equivalent amounts of this radioactive compound.

Distribution

About 80-95% of Δ^9-THC becomes bound in vitro to human plasma proteins, predominantly those in the lipoprotein fraction (13,14). A metabolite of Δ^9-THC, 11-OH-Δ^9-THC also binds to plasma proteins (94-99%) (15), but interestingly it becomes bound primarily to both the albumin and α-lipoprotein fractions.

In man, Lemberger et al. (16,17) investigated the plasma concentrations of intravenously administered ^{14}C-Δ^9-THC. In volunteers, who claimed no previous exposure to cannabis, Δ^9-THC disappeared rapidly from plasma during the first few hours; then, it disappeared more slowly. The apparent half life of Δ^9-THC was estimated to be about 56 hours. The initial, rapid phase was attributed to distribution of Δ^9-THC from the intravascular compartment to the tissue compartment, as well as to metabolism and excretion of the compound. When this study was repeated in subjects who had smoked marihuana daily for at least one year prior to the experiment, a biphasic plasma disappearance curve was observed again. However, the apparent half life of the drug was estimated to be only 27 hours. There were no group differences in the apparent volumes of distribution of Δ^9-THC. Except for the fact that one group smoked marihuana chronically, the subjects were similar with respect to age, weight, and cigarette (tobacco) smoking habits. It appears that a constituent of the marihuana cigarette (whether Δ^9-THC itself, another cannabinoid, or a polycyclic hydrocarbon) is absorbed during the smoking process and induces enzymes that catalyze the disappearance of Δ^9-THC from human plasma.

In marihuana smokers, Δ^9-THC disappears from plasma similarly after inhalation and after intravenous administration (10,12,18). Δ^9-THC and its metabolites were detected in human plasma for at least three days after single-dose administration of Δ^9-THC by either route. These findings suggest that Δ^9-THC and its metabolites are stored in human tissues for prolonged periods. Indeed, the persistence of this drug and its metabolites in the tissues has been reported in animal studies. High tissue concentrations of Δ^9-THC would reflect its marked lipid solubility as well as binding to tissue protein. Furthermore, in man a high degree of penetration of Δ^9-THC into tissues is indicated by an absurdly large apparent volume of distribution, about 500 liters (11). However, when one considers the distribution of total radioactivity after ^{14}C-Δ^9-THC administration, the apparent volume of distribution approaches that of the total body water (about 50 liters). This suggests that the metabolites of Δ^9-THC are considerably less lipid soluble than the parent compound and are not

sequestered in tissue to any great extent. The marked sequestration of Δ^9-THC in animal tissues is of particular interest because the highest concentrations of Δ^9-THC (and its metabolites) are found in the lung, that organ which receives the initial drug "bolus" when smoke is inhaled by man from a marihuana cigarette.

Metabolism and Excretion of Tetrahydrocannabinols

After the intravenous administration of ^{14}C-Δ^9-THC to man, 11-OH-Δ^9-THC represented about 20% of the administered dose of radioactivity recovered in the feces, and the 8,11-dihydroxy compound represented slightly less (11,19). Also present in feces were polar metabolites that perhaps were conjugates of the hydroxylated metabolites. Human feces did not contain detectable amounts of acidic metabolites of Δ^9-THC (11).

About 20-30% of the radioactivity from a dose of ^{14}C-Δ^9-THC appears in human urine after oral and intravenous administration; essentially all this urinary radioactivity is in the form of metabolites of Δ^9-THC. Only about 5% of the urinary radioactivity was attributable to 11-OH-Δ^9-THC (16). About 90% of the urinary metabolites were polar and acidic. These are characteristics of 11-carboxylated metabolites of Δ^9-THC found initially in rabbit urine (20), and later identified in human urine after Δ^9-THC administration (19).

After the intravenous administration of ^{14}C-Δ^9-THC to human marihuana abstainers, 67% of the administered dose of radioactivity was excreted in one week (22% in urine and 45% in feces) (16). In chronic marihuana smokers, 71% of the total dose was recovered in the excreta, but a significantly greater proportion of radioactivity was excreted in urine compared to the nonusers (17). Similar patterns of Δ^9-THC elimination were reported after oral administration to chronic users (9,10).

The rate of elimination of Δ^9-THC from different species appears to correlate well with the plasma half lives of the drug; for example, in rabbits the half life of the drug is short and it is rapidly metabolized and excreted; whereas in man the half life of Δ^9-THC is long and it is metabolized and excreted more slowly.

11-OH-Δ^9-THC is formed rapidly from Δ^9-THC in man, appearing in plasma within 10 minutes after the intravenous administration of Δ^9-THC (16). After the intravenous administration of tritiated 11-OH-Δ^9-THC to infrequent marihuana users, the drug disappeared from plasma in a biphasic fashion with an apparent half life of about 22 hours (21). The physiologic disposition of 11-OH-Δ^9-THC and Δ^9-THC are similar. The 8,11-dihydroxy is a metabolite of both Δ^9-THC and 11-OH-Δ^9-THC. Also, both the 11-OH-Δ^9-THC and 8,11-dihydroxy compound are eliminated primarily via the feces (19,22). When 11-OH-Δ^9-THC was administered to man, about 22% was excreted in urine and 50% in feces. In addition, the metabolites of 11-OH-Δ^9-THC present in human urine and feces were both qualitatively and quantitatively the same as those seen after Δ^9-THC administration (19,22).

Is the Pharmacologic Activity Due to Δ^9-THC or an Active Metabolite?

In chronic cannabis smokers, indirect evidence suggests that metabolites of Δ^9-THC may be partly responsible for Δ^9-THC activity (10,11,12,23,24,25). After the oral administration of Δ^9-THC and at the time of peak psychologic effect, plasma concentrations of Δ^9-THC are low compared to concentrations of its metabolites. At equivalent plasma concentrations of Δ^9-THC the pharmacologic effects observed after oral administration exceed those seen after rapid I.V. administration (11). When Δ^9-THC was inhaled, its concentration in plasma was initially high, but it was the concentrations of the metabolites in plasma that showed a temporal correlation with the psychologic effects.

Although in man the plasma concentrations of 11-OH-Δ^9-THC were reportedly lower than those of other metabolites of Δ^9-THC (9,16), plasma concentrations may not necessarily reflect concentrations in brain. For example, in mice, the concentrations of 11-OH-Δ^9-THC in brain are about eight times those in plasma (26). Ryrfeldt et al. (27) also reported a greater tendency for the 11-hydroxy metabolite of Δ^9-THC to distribute into mouse brain and other tissues compared to Δ^9-THC itself. Likewise, Perez-Reyes et al. (28) reported that 11-OH-Δ^9-THC penetrated the brain of mice four times faster than Δ^9-THC.

Recent studies by Lemberger and coworkers (21,22) provide evidence that 11-OH-Δ^9-THC may play a direct role in the actions of Δ^9-THC in man. After the intravenous administration of 1 mg of either 11-OH-Δ^9-THC or Δ^9-THC, there was a marked increase in subjective symptoms, a pronounced psychologic "high", and a marked tachycardia. The effects from 11-OH-Δ^9-THC were rapid in onset and were about twice the intensity of an equivalent dose of Δ^9-THC. The finding that the metabolic fate of 11-OH-Δ^9-THC was both quantitatively and qualitatively similar to that seen after the administration of Δ^9-THC suggest that after the administration of marihuana or hashish, Δ^9-THC is rapidly converted in man to 11-OH-Δ^9-THC which is responsible in part for the psychopharmacologic effect. Hollister (29) also studied the activity of intravenously administered 11-OH-Δ^9-THC in man and found it to be about 1.25 times more potent than Δ^9-THC. Perez-Reyes et al. (30) also found 11-OH-Δ^9-THC to be active in subjects after a slow intravenous infusion (1 mg/5 min); however, they did not observe major differences in potency between Δ^9-THC and the 11-OH compound. Perhaps the different techniques used in these studies, particularly the rates of drug administration, are responsible for the differences between these and the above-mentioned findings.

Summary and Conclusions

In the past, it has been difficult to detect Δ^9-THC (or cannabis) in biologic fluids and excrement of humans after the usage of marihuana. Among the reasons for these difficulties were: 1) the low dose of drug used to produce the desired psychologic effects in man; 2) the low plasma concentrations due to a high degree of distribution into tissues; 3) predominant excretion of Δ^9-THC via the feces, which is not assayed routinely; and 4) the extensive metabolism of Δ^9-THC such that very little unchanged drug appears in urine. In addition, the excretion of high

concentrations of an acidic metabolite of Δ^9-THC has made it difficult to selectively quantitate Δ^9-THC in urine. In recent years the availability of ^{14}C-labeled Δ^9-THC and the development of sensitive and specific methods (GLC/mass spectroscopy) for the detection and quantitation of this drug and its metabolites have led to significant progress in the elucidation of the physiologic disposition of this compound. In brief, Δ^9-THC appears to be readily absorbed from the lung when inhaled in smoke from a marihuana cigarette. Once absorbed, the drug may become bound to plasma lipoprotein as well as to tissue protein. Δ^9-THC is metabolized by the liver to 11-OH-Δ^9-THC, 8,11-dihydroxy-Δ^9-THC, 11-carboxylated compounds, and other unidentified polar compounds. The 11-OH-Δ^9-THC has been implicated as a pharmacologically active metabolite of Δ^9-THC. The parent drug and its hydroxylated metabolites are eliminated from the body via the feces and, to a lesser degree, via the urine.

The continued development of methods for the precise measurement of the cannabinoids in biological specimens should eventually permit identification of other metabolites of Δ^9-THC formed by man. Also, it should be of some interest to assess the potential interactions between Δ^9-THC, its metabolites, and unrelated drugs that may share common pathways of metabolic disposition. For example, it would be important to know whether chronic use of Δ^9-THC alters the disposition (hence the pharmacologic/toxicologic effects) of commonly used therapeutic agents (or other drugs of abuse) that are eliminated metabolically.

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Marihuana: Studies on the Disposition and Metabolism of Delta-9-Tetrahydrocannabinol in Man

Abstract. *Δ^9-Tetrahydrocannabinol (the major active component of marihuana) administered intravenously to normal human volunteers persists in plasma for more than 3 days ($t_{1/2}$=56 hours). Its metabolites appear in plasma within 10 minutes after administration and persist along with the precursor compound: Δ^9-Tetrahydrocannabinol is completely metabolized in man, and the radioactive metabolites are excreted in urine and feces for more than 8 days.*

Marihuana and hashish are psychoactive plant materials prepared from *Cannabis sativa*. The active component of *Cannabis* in animals (*1*) and in man (*2*) has been reported to be Δ^9-tetrahydrocannabinol (Δ^9THC). Until recently there has been little information regarding the metabolism and disposition of Δ^9THC because of the difficulty in synthesizing and assaying this com-

pound. The availability of ^{14}C-labeled Δ^9THC of relatively high specific activity has made possible studies of its physiologic disposition and metabolism in rabbits (*3*) and rats (*4*). However, studies on the physiologic disposition and metabolism in man have not been reported. We now report the levels of Δ^9THC in plasma after its intravenous injection, its retention in body stores, and the excretion of its metabolites in man.

Three normal volunteers (one male and two females) between the ages of 18 and 22 who professed no previous exposure to *Cannabis* (*5*) were given 0.5 mg of [^{14}C]Δ^9THC (*6*) intravenously. Blood samples were obtained at intervals thereafter, and urine and feces were collected for up to 10 days after injection of the labeled compound. The unchanged Δ^9THC was measured by extraction at *p*H 6.5 to 7.5 into four volumes of heptane containing 1.5 percent isoamyl alcohol.

The radioactivity in the organic phase was assayed by liquid-scintillation spectrometry. Of the Δ^9THC added to plasma or urine 95 ± 5 percent was recovered. Total radioactivity in aliquots of plasma and urine and in a methanol extract of feces was determined by liquid-scintillation spectrometry. After extraction of Δ^9THC with heptane, polar metabolites which remained in the aqueous phase were extracted first with ether and then with ethyl acetate. The most polar metabolites and conjugates remained in the aqueous phase.

After intravenous administration of [^{14}C]Δ^9THC the amount of this compound in plasma declined rapidly during the first hour (with a half-life of about 30 minutes) (Fig. 1). After 1 hour the Δ^9THC fell much more slowly (with a half-life of 50 to 60 hours). To establish the identity of the apparent Δ^9THC measured in the heptane extract of plasma, samples of plasma collected during the first hour, during the remainder of day 1, and during days 2 and 3 were pooled separately and extracted with heptane. After evaporation at reduced pressure, the residue was taken up in a small volume of ethanol and applied to an Eastman silica-gel chromatogram sheet for development in a hexane : acetone system (3:1). Most of the radioactivity (about 85 percent) in the extract obtained during the first hour is chromatographically identical with authentic Δ^9THC (Fig. 2). A small portion of the radioactivity in the heptane extract had the mobility of authentic 11-OH-THC (*7*). Almost all of the radioactivity in the heptane extract of plasma obtained during the remainder of day 1 and during days 2 and 3 had the same mobility on chromatography as synthetic Δ^9THC.

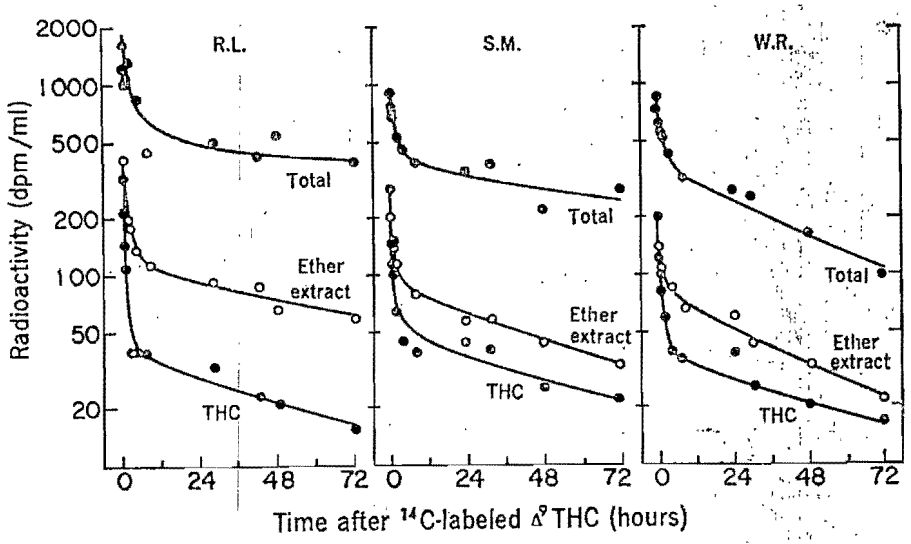

Fig. 1. Plasma levels of Δ^9THC, total radioactivity, and ether-extractable radioactivity after the intravenous injection of [^{14}C]Δ^9THC (0.5 mg in 1 ml of ethanol) to three normal volunteers. The radioactive solution was injected during an interval of 1 minute into the tubing of a rapidly flowing intravenous infusion of 5 percent dextrose in water. The dose ranged from 80 to 146 nc/kg (5.6 μg/kg to 7.9 μg/kg). Blood samples were drawn in heparinized syringes from the opposite arm at various times. Plasma was assayed for Δ^9THC, total radioactivity, and ether-extractable radioactivity by liquid-scintillation spectrometry.

The decline of total radioactivity and the more polar ether-extractable metabolites in plasma was similar to that of Δ^9THC. A rapid initial decline preceded a much slower phase of disappearance from the plasma. Polar metabolites were formed rapidly and were present in plasma at higher concentrations than Δ^9THC.

About 30 percent of the administered radioactivity was excreted in the urine (Fig. 3); most appeared during day 1, but metabolites continued to be excreted into the urine for more than 1 week. Less than 1 percent of the urinary radioactivity was unchanged Δ^9THC, and ^{14}C-labeled 11-OH-THC did not appear to account for more than a small percentage of the metabolites. Even after hydrolysis of conjugates with Glusulase (*8*), which increased the proportion of labeled 11-OH-THC in the

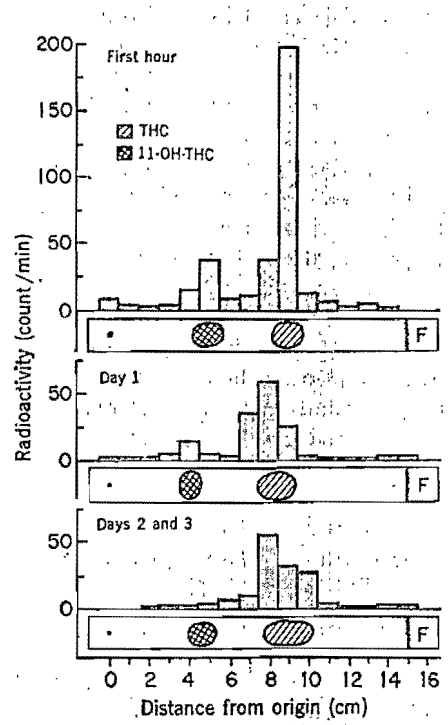

Fig. 2. Histogram of thin-layer chromatography of ^{14}C-labeled Δ^9THC extracted from plasma at various times. R.L. represents a typical subject. Samples of plasma from the first hour, from the remainder of day 1, and from days 2 and 3 were pooled separately and extracted with four volumes of a solution of heptane containing 1.5 percent isoamyl alcohol. The extract was evaporated to dryness at reduced pressure, dissolved in a small volume of ethanol, applied to an Eastman silica-gel chromatogram sheet, and developed in a hexane : acetone (3 : 1) system. Authentic Δ^9THC and 11-OH-Δ^9THC were cochromatographed with the heptane extract. The sheet was cut into 1-cm strips from the origin to the solvent front and placed in vials containing scintillation solution, and radioactivity was determined by liquid-scintillation spectrometry.

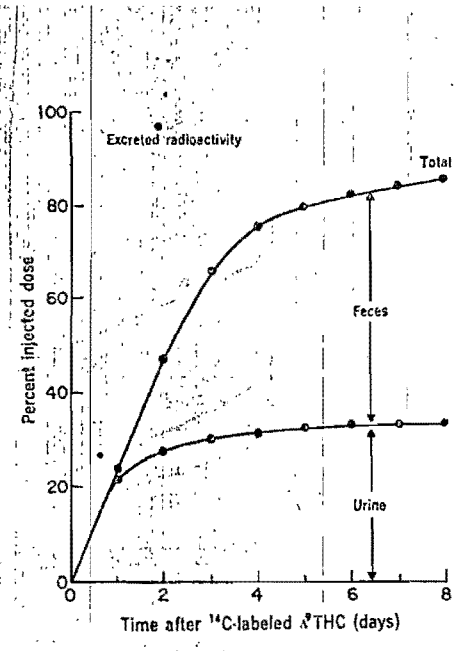

Fig. 3. Cumulative excretion of radioactivity after the intravenous injection of [^{14}C]Δ^9THC. W.R. represents a typical subject. Urine and feces were collected for at least 8 days after the intravenous administration of [^{14}C]Δ^9THC. Urine and feces were frozen until analyzed. The feces were suspended in three volumes of methanol and vigorously shaken for 10 minutes on a mechanical shaker. The material was centrifuged, and an aliquot of the methanol extract was assayed for total radioactivity. Urine was assayed directly for total radioactivity by liquid-scintillation spectrometry.

ether extract, about 80 percent of the metabolites remained uncharacterized. The extraction and chromatographic properties of this portion suggested that the radioactivity might be in the form of polar compounds.

About one-half of the radioactivity administered as [^{14}C]Δ^9THC is recovered in the feces (Fig. 3). There was almost no radioactivity in the feces collected on day 1, presumably because the compounds were excreted in the bile and their passage through the gastrointestinal tract delayed excretion in the feces. A methanol extract of feces containing the radioactivity was evaporated at reduced pressure, and the residue was dissolved in phosphate buffer (0.1*M*, *p*H 6.5). The heptane extract of this aqueous solution contained most of the radioactivity. Almost none had the chromatographic characteristics of Δ^9THC, however; and only 10 percent appeared to be the 11-hydroxy derivative. Hydrolysis of conjugates by incubation with Glusulase increased the apparent 11-OH-[^{14}C]THC to about 20 percent of the radioactivity. Most of the remaining ^{14}C stayed at the origin, presumably in the form of more polar metabolites.

The initially rapid decrease of [^{14}C]Δ^9THC in the plasma represents redistribution of the Δ^9THC from the intravascular compartment into tissues (including brain) and metabolism. In man the effects of marihuana are maximum within 15 minutes, diminished between 30 minutes and 1 hour, and largely dissipated by 3 hours (*9*). This would be consistent with the finding that, after intravenous administration of [^{14}C]Δ^9THC, the concentration in plasma rapidly declines, with a half-life of about 30 minutes, and that over two-thirds of the total radioactivity excreted in the urine is present during day 1. During this initial phase, metabolites of Δ^9THC are present in higher concentrations in the plasma than the parent drug (Fig. 1).

The slower decline of Δ^9THC in plasma ($t_{1/2} = 56$ hours) and of total radioactivity ($t_{1/2} = 67$ hours) presumably represents retention and slow release of the drug from its stores. Since Δ^9THC is a nonpolar compound, it may accumulate in fat or other tissues such as lung which have an affinity for drugs. It has been reported that, in animals (*3, 10*) soon after the intravenous administration of labeled Δ^9THC, higher levels of radioactivity were present in lung than in other tissues. If, indeed, the Δ^9THC is bound in lung, then in man this would be even more significant since inhalation is the usual route of administration.

The finding that Δ^9THC and its metabolites persist in humans for long periods indicates that the drug and its metabolites accumulate in tissues when administered repeatedly. It may explain in part the phenomenon of "reverse tolerance" seen in chronic users of marihuana. Possibly a critical degree of tissue saturation must be attained before effective threshold levels of Δ^9THC can be achieved. On the other hand, long-term administration of marihuana may induce enzymes which convert the drug to an active metabolite of Δ^9THC. In animals it appears that 11-OH-THC is as active as Δ^9THC (*7*). In the present study of naive subjects, 11-OH-THC appears to be only a minor metabolite of the Δ^9THC. However, the more polar metabolites present in urine and feces may represent further metabolic products of 11-OH-THC.

Since a considerable percentage of the metabolites of Δ^9THC are excreted in urine during day 1 after its administration, it should be possible, by means of solvent extraction and thin-layer chromatography, to develop a sensitive assay for the detection of metabolites of Δ^9THC in human urine.

From our results it can be concluded that Δ^9THC persists for a long time in normal volunteers and that it is completely metabolized in man and excreted as polar metabolites in urine and feces.

LOUIS LEMBERGER
STEPHEN D. SILBERSTEIN
JULIUS AXELROD
IRWIN J. KOPIN

Laboratory of Clinical Science, National Institute of Mental Health, Bethesda, Maryland 20014

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5. The volunteers were in residence at the Clinical Center, National Institutes of Health, for periods of at least 1 month prior to the study and received no medications either during this period or during our study.
6. The [^{14}C]Δ^9THC was synthesized by Research Triangle Institute (Research Triangle Park, North Carolina; sample JW-III-47A; specific activity (17.5 μc/mg, 5.5 mc/mmole) is labeled on the 2- and 4-positions of the benzene ring. Purity was shown to be greater than 99 percent by chromatography. The radioactive material was subjected to pyrogen and sterility tests and then prepared sterilely in single-dose vials in ethanol by the NIH Radiopharmacy.
7. 11-Hydroxytetrahydrocannabinol has been synthesized independently by several investigators: E. B. Truit, *Fed. Proc.* 29, 619 (1970); R. L. Foltz, A. F. Fentiman, Jr., E. G. Leighty, J. L. Walter, H. R. Drewes, W. E. Schwartz, T. F. Page, E. B. Truitt, Jr., *Science* 168, 844 (1970); M. E. Wall, D. R. Brine, G. A. Brine, C. G. Pitt, R. I. Freudenthal, H. D. Christensen, *J. Amer. Chem. Soc.* 92, 3466 (1970); Z. Ben-Ziv, R. Mechoulam, S. Burstein, *J. Amer. Chem. Soc.* 92, 3468 (1970); I. M. Nilsson, S. Agurell, J. L. G. Nilsson, A. Ohlsson, F. Sandberg, M. Wahlqvist, *Science* 168, 1228 (1970). In our study 11-hydroxy-Δ^8THC was used as a reference compound since 11-hydroxy-Δ^9THC is not commercially available.
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11. The ^{14}C-labeled Δ^9THC, the unlabeled Δ^9THC, and the unlabeled 11-OH-Δ^8THC were obtained from Drs. J. A. Scigliano and M. Braude, Center for Studies of Narcotics and Drug Abuse, National Institute of Mental Health, Chevy Chase, Maryland.

28 September 1970

Effects of Marijuana and Tobacco Smoke on DNA and Chromosomal Complement in Human Lung Explants

HUMAN lung explants exposed to smoke from marijuana or from Kentucky Standard tobacco cigarettes have been reported to display abnormalities of cell morphology, mitosis, DNA synthesis and atypical proliferation[1]. We report here a study designed to test the effects of both types of smoke on the DNA and chromosomal complement.

We used the model system for exposing lung explants to puffs of fresh smoke in standardized conditions[2]. Lung explants were prepared and exposed to cigarettes made from marijuana or Kentucky Standard tobacco as previously described[1]. Normal lung tissue was obtained not only from older patients operated on for pulmonary tumours[1], but also from a healthy young man (age 25 yr) killed in an accident. The DNA determinations were carried out in metaphases and telophases of the original stained lung cultures by Feulgen microfluorometry[3]. All together, over 2,000 cells were examined in control cultures and correspond-

ing cultures from 4–70 days after exposure to marijuana or tobacco cigarette smoke. Chromosomes were prepared according to Inui *et al.*[3] for the examination of chromosomal status.

Results were reproducible in all cultures. They were essentially the same for cultures derived from the lungs of the older tumour patients and from those derived from the healthy young man. There was a good agreement between chromosomal and DNA data.

Table 1 Comparison between the DNA Content* in Metaphases and Telophases†, and Number of Chromosomes‡ in Fibroblastic Cells of a Control Adult Human Lung Explant and after Exposure to Fresh Smoke from Marijuana and Kentucky Standard Cigarettes

Type of experiment	Mean frequency in per cent: DNA content in metaphases		DNA content in telophases		Number of chromosomes	
	4 DNA	≷ 4 DNA	2 DNA	≷ 2 DNA	2 N	≷ 2 N
Control	76	24	70	30	56	44
Kentucky Standard	56	44	48	52	36	64
	pCo = 0.01		pCo < 0.0065		pCo < 0.0005	
Marijuana	52	48	52	48	31	69
	pCo = 0.0025		pCo < 0.005		pCo < 0.0005	

* Microfluorometry.
† 431 cells measured.
‡ 633 metaphases counted.

There were significant differences between control and exposed cultures; 4–28 days after exposure to marijuana or Kentucky tobacco cigarettes there was not only a significant decrease in number of cells with 2 N, and with 4 DNA and 2 DNA (Table 1), but the variability of cells with deviating DNA content and chromosomal numbers was greater than that observed in controls (Figs. 1 and 2). This difference was especially marked after marijuana cigarette smoke.

It thus seems that exposure of human lung explants to fresh smoke from marijuana or Kentucky Standard tobacco cigarettes evokes not only abnormalities in DNA synthesis, mitosis and growth[1], but also results in alterations of DNA and chromosomal complement, that is in a disturbance of the genetic equilibrium of the cell population. The finding that these changes were

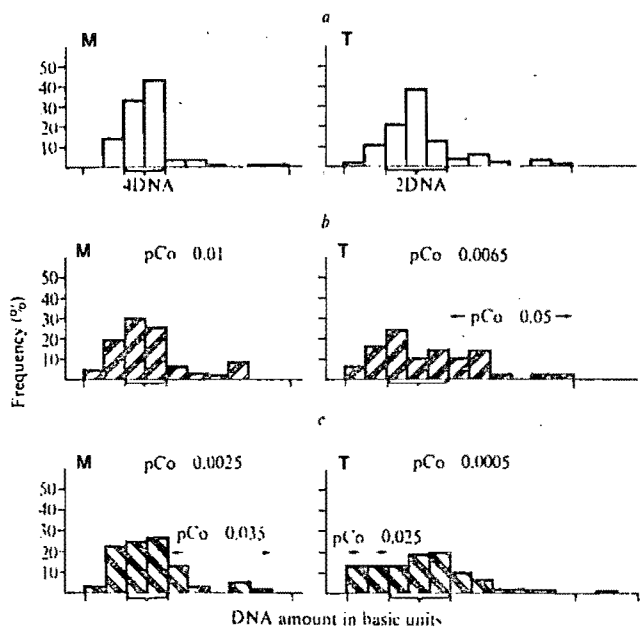

Fig. 1 Comparison between the DNA content (Feulgen microfluorometry) in metaphases (M) and telophases (T) of fibroblastic cells (number measured = 431) from (*a*) control adult human lung explant, and after exposure to fresh smoke from (*c*) marijuana, and (*b*) Kentucky Standard cigarettes (5 experiments).

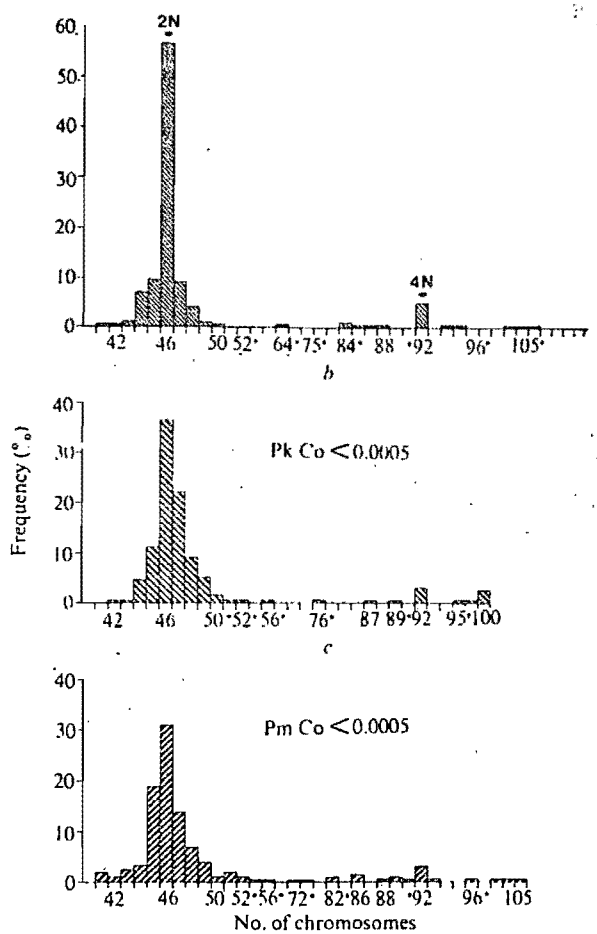

Fig. 2 Comparison between the number of chromosomes of fibroblastic cells (number of metaphases counted = 633) from (*a*) control adult human lung explant and after exposure to fresh smoke from (*c*) marijuana and (*b*) Kentucky Standard cigarettes (number of cultures examined = 12).

observed very early, and that they persisted for prolonged periods after exposure, indicates that these alterations are not lethal to the cells. The question whether the cells with abnormal DNA and chromosomal complement are responsible for the subsequent atypical growth, and may represent an early stage preceding malignant transformation, cannot be answered at present.

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C. LEUCHTENBERGER
R. LEUCHTENBERGER
U. RITTER

Swiss Institute for Experimental Cancer Research, Lausanne

N. INUI

Department of Experimental Pathology, Cancer Institute, Tokyo

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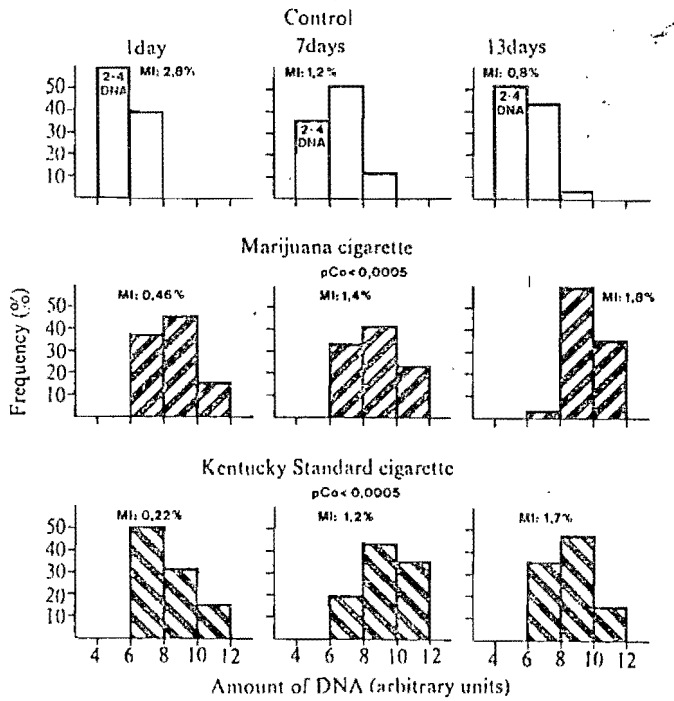

Fig. 1 Comparison between effects of fresh smoke from marijuana and Kentucky Standard cigarettes on the DNA content of fibroblastic cells in human adult lung explants, one, seven and thirteen days after exposure (Feulgen microspectrography). 375 cells measured. MI: Mitotic index.

Effects of Marijuana and Tobacco Smoke on Human Lung Physiology

Mouse lung explants exposed to smoke from cigarettes to which marijuana was added have been reported to display more cellular abnormalities than those exposed to smoke from cigarettes without marijuana[1]. We report here a study designed to test the effects of smoke from cigarettes made of marijuana only on human lung explants, and to compare these effects with those obtained after exposure to smoke from Kentucky Standard cigarettes.

We used the model system developed for preparing and exposing lung explants to puffs of fresh smoke in standardized conditions[2]. Fresh human adult lung tissue was obtained from men (45–56 yr) who had undergone surgery because of pulmonary tumours. For the lung explants pieces of "normal lung" were taken far distant from the tumour. Absence of tumour was established by microscope examination. The cultures, which at three to four weeks showed a regular monolayer of fibroblastic cells, were exposed in a 'Filtrona CSM 12' smoking machine to puffs of fresh smoke from marijuana cigarettes, or to puffs of whole fresh smoke, or smoke of the gas-vapour phase[2] from Kentucky Standard cigarettes. Marijuana cigarettes containing 1.8 g of marijuana (UNC 303, 0.6% tetrahydrocannabinol (THC)) for each cigarette were prepared with the same paper as that for Kentucky Standard tobacco.

Experiments were carried out on over 1,300 cultures of human lung explants. For each experiment a minimum of eight sets consisting of twenty-four coverslips with matched lung explants was used. Each set comprised a control culture not exposed to cigarette smoke, a culture exposed to four puffs per day (25 ml. at intervals of 58 s) of fresh smoke from marijuana cigarettes for 4–10 consecutive days, and a culture exposed in the same manner to two puffs from Kentucky Standard cigarettes. The lower number of puffs from Kentucky Standard cigarettes was given because of the relatively high cytotoxic effect of this type of cigarette on the cultures. The larger puff volume of 25 ml., instead of 8 ml. used in the previous study[1], was chosen because it resembled more closely the standard puff volume of 35 ml. inhaled by human smokers. Media were changed immediately after each exposure.

Fig. 2 *a*, Metaphase of fibroblastic cell in human adult lung explant, 6 days after 4 exposures to 4 puffs of marijuana cigarette smoke (H and E, × 1,000). Note tripolar metaphase with pieces of chromosomes lying distant from metaphase and the very large spindle. *b*, Metaphase of fibroblastic cell in human adult lung explant, 6 days after 4 exposures to 2 puffs of Kentucky Standard cigarette smoke (H and E, × 1,000). Note chromosome distant from main metaphase. *c*, Same culture as *b*. Anaphase, note lagging of chromosomes.

Chromosomal behaviour during mitosis was studied in live cultures* under phase contrast and in the original stained cultures from 1–45 days after exposure. The mitotic index was obtained by counting all mitotic figures and relating them to the total number of cells of each monolayer grown on the coverslips. Frequency of mitotic abnormalities was obtained by counting all abnormal mitoses and relating them to the total number of mitosis in each culture. DNA metabolism was examined by autoradiography and Feulgen microspectrography[2–4].

Results were reproducible in all cultures. There were significant differences between control cultures and those exposed to smoke from marijuana and Kentucky Standard cigarettes (Table 1). From 1–4 days after exposure, cytotoxic effects such as pyenosis, necrosis and cell death were observed in the exposed cultures, accompanied by a striking decrease of mitosis and DNA synthesis. These early alterations were more marked after exposure to either whole smoke or the gas vapour phase of Kentucky Standard than they were after smoke from marijuana cigarettes. The lesser cytotoxic effect of marijuana, which contains a sticky resin, may perhaps be because cigarettes made from marijuana have a larger side-stream than Kentucky Standard cigarettes so that, in spite of the higher dose, less smoke *per se* reached the cultures[1]. Other alterations observed in surviving cells were essentially the same after exposure to marijuana and Kentucky Standard cigarettes. There was a marked increase in size of cytoplasm, nucleoli and nuclei[1], accompanied by a significant increase in DNA content (Fig. 1). In control cultures even the largest nuclei contained 2–4 or only slightly higher DNA amounts, in all exposed cultures none of the large nuclei had 2–4 DNA, but the majority carried a significantly higher DNA content ($P=0.0005$). As can be seen from Table 1 and the example in Fig. 1, this striking increase in DNA was observed very early after exposure (1 day) at a period where mitosis and DNA synthesis were inhibited, and at later periods after exposure (7–45 days), at a period where stimulation of mitosis and DNA synthesis occurred (Table 1, Fig. 1). In addition, from 1–45 days after exposure, all cultures displayed mitotic abnormalities, which were not only more severe, but had also a significantly higher frequency than in control cultures. The abnormal behaviour of chromosomes during division was particularly clear in metaphase and anaphase, in which lagging and breakage of chromosomes and occurrence of tripolar metaphases and very large spindles were observed in living and fixed cultures (Fig. 2*a-c*). The difference in frequency of mitotic abnormalities in exposed and control cultures was particularly evident in young cultures. These control cultures had 0–3% abnormal mitosis, and the corresponding exposed cultures had 20–30%, that is a mean increase of about 7 times (Table 1). In older exposed cultures the significantly higher frequency of mitotic abnormalities persisted. Since, however, the frequency of mitotic abnormalities increased with

the frequency of mitotic abnormalities [illegible] controls (up to 10%), the difference, although still statistically significant, was somewhat less than for

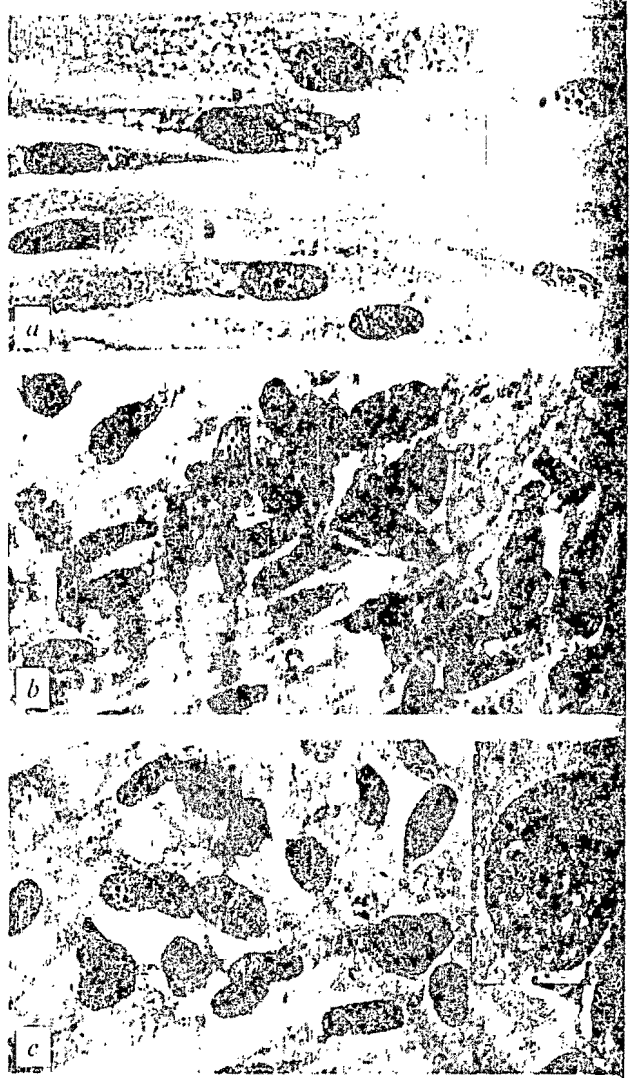

Fig. 3 *a*, Fibroblastic cells in monolayer in control culture of human adult lung explants (H and E, ×500). Note well separated cells. *b*, Same culture as *a*, but 17 days after 4 exposures to 4 puffs of marijuana cigarette smoke (H and E, ×500). Note irregularity and disparity of cells, and criss-cross formation. *c*, Same culture as *a*, 17 days after 4 exposures to 2 puffs of Kentucky Standard cigarette smoke (H and E, ×500). Note essentially the same irregularity of culture as in *b*. Note also irregular shape of large nucleus, multiple nucleoli (inset ×1,000)

Table 1 Comparison of Effects of Fresh Smoke from Marijuana and Kentucky Standard Cigarettes on Mitosis and Metabolism in Fibroblastic Cultures of Adult Human Lung Explants ($n_1=200$)

Type of cigarette	Time after last exposure (in days)	Type and mean ratios of frequency of alterations $\frac{\text{exposed cultures}}{\text{controls}}$							
		Mitotic index		DNA synthesis (^{3}H-TdR) (number of labelled cells)		DNA content * (number of cells with high DNA content) $n_2=7{,}500$		Mitotic abnormalities	
		Decrease	Increase	Decrease	Increase	Decrease	Increase	Decrease	Increase
Marijuana	1–4	4±0.2 pCo=0.001	—	3±0.1 pCo=0.001	—	—	5±0.5 pCo>0.0001	—	7±1.7 pCo>0.000
	7–45	—	2±0.2 pCo=0.005	—	2±0.2 pCo=0.005	—	5±0.5 pCo>0.0001	—	3±0.6 pCo=0.00
Kentucky Standard whole smoke	1–4	9±0.2 pCo=0.0005	—	10±0.2 pCo>0.0001	—	—	5±0.5 pCo>0.0001	—	7±1.5 pCo>0.000
gas–vapour phase	7–45	—	2±0.2 pCo=0.005	—	2±0.2 pCo=0.005	—	5±0.5 pCo>0.0001	—	3±0.5 pCo=0.00

n_1=No. of cultures examined. n_2=No. of cells measured. *Feulgen microspectrography.

cultures (Table 1). A detailed study of time sequential microscopic, ultrastructural and cytochemical effects of marijuana and tobacco smoke on young and old human adult and foetal lung cultures has been completed (Leuchtenberger, C. *et al.*, in preparation).

The marked decrease of DNA synthesis and mitosis observed early after exposure was followed at later periods (7–45 days) by an increase of mitosis and DNA synthesis (Table 1). In comparison with controls there was about a two-fold augmentation of these and this was accompanied by irregular growth, characterized by disparity in shape and size of nuclei and nucleoli, piling up, and criss-cross formations of cells. The appearance of exposed cultures was strikingly different from the corresponding control cultures, which showed rather regular monolayers with more or less contact inhibition (Figs. 3*a–c*).

It therefore seems that fresh smoke from cigarettes made from marijuana evokes essentially the same abnormalities in DNA metabolism, mitosis and growth in fibroblastic cells of human adult lung explants as does whole fresh smoke or the gas-vapour phase from Kentucky Standard cigarettes. This similarity is particularly noteworthy when the important role of cigarette smoke for pulmonary carcinogenesis is examined[5,6], but while it is tempting to suggest that smoke from marijuana cigarettes plays the same role in pulmonary carcinogenesis, no conclusions can be drawn until results of epidemiological studies in human marijuana smokers and chronic inhalation experiments in laboratory animals with marijuana cigarettes become available.

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CECILE LEUCHTENBERGER
RUDOLF LEUCHTENBERGER
ANDRÉE SCHNEIDER

Swiss Institute for Experimental Cancer Research,
Lausanne,
Switzerland

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Inhibition of Cellular Mediated Immunity in Marihuana Smokers

Abstract. *The cellular mediated immunity of 51 young chronic marihuana smokers, as evaluated by the lymphocyte response in vitro to allogeneic cells and to phytohemagglutinin, was significantly decreased and similar to that of patients in whom impairment of T (thymus derived) cell immunity is known to occur. This inhibition of blastogenesis might be related to an impairment of DNA synthesis.*

It has been previously reported (*1*) that delta-9-tetrahydrocannabinol (Δ^9-THC), a psychoactive substance of *Cannabis*, when administered to rodents alters their cellular mediated immune responsiveness, and it was suggested that similar changes might also occur in man. In our study the mixed lymphocyte culture (MLC) and phytohemagglutinin (PHA) responsiveness of 51 marihuana smokers, 16 to 35 years old (median age 22), were studied. Only subjects who had used *Cannabis* products (at the exclusion of other drugs) at least once a week (average four times a week) for at least 1 year (average 4 years) were selected for this investigation.

Eighty-one healthy volunteers, 20 to 72 years of age (median age 44) were used as controls. Purified lymphocyte suspensions were prepared from fresh samples of venous blood by the Ficoll-Isopaque density gradient method (*2*). A microculture system was used for screening of cellular responsiveness (*3*). For the MLC test, 1×10^5 responding cells were incubated, per well, with 2×10^5 stimulating cells pooled from a panel of ten donors, phenotypically different [allogeneic cells in which 25 different HL-A specificities were represented (*4*)].

For the PHA test, 2×10^5 responding cells were incubated per well with 1 μg of purified PHA. The medium used was RPMI 1640 with penicillin, streptomycin, and glutamine, to which 25 percent autologous serum was added.

Results are summarized in Table 2 and compared with data obtained in 60 patients with cancer, 20 patients with uremia, and 24 renal allograft recipients with iatrogenically induced immunosuppression. The mean values registered in the group of marihuana users were significantly lower than those of the normal, but much older, control group. Since an inverse correlation exists between cellular immunity, as reflected by in vitro lymphocyte blastogenesis and aging (*5*), results obtained in the group of marihuana smokers may be interpreted as being indicative of cellular hyporesponsiveness. Supporting this conclusion is the close similarity between the depressed MLC and PHA responsiveness of marihuana users and that of cancer (*6*), uremia (*7*), and immunosuppressed transplant patients in whom impairment of T (thymus derived) cell immunity is known to occur. Furthermore, we observed that in vitro inhibition of PHA-induced blastogenesis of normal human lymphocytes started with 1.6 μM THC and was complete with 20 μM.

The major psychologically active constituent of *Cannabis sativa* is Δ^9-THC. This substance, as well as its metabolites, is insoluble in H_2O, but is very fat soluble, and has a half-life of several days in tissues where it might exert a cumulative and pharmacological effect (*8*). Such an effect might be related in a still unknown way to the depressed cellular immune response in vitro of chronic marihuana smokers. The effect of THC on adrenergic receptors (*9*) might also play a role in its immunosuppressive activity, as was suggested for other drugs administered continuously over a long period (*10*).

This inhibition of blastogenesis might result from an impairment of DNA synthesis. One of us (A.M.) sampled lymphocytes from four marihuana smokers, cultivated the cells for 72 hours, and then observed a decreased number of cells during the period of DNA synthesis (S period of the cell cycle). There was also an increased incidence of chromosomal breakages,

Table 1. Comparative cellular mediated immunity of normal subjects, marihuana smokers, and patients with impairment of T cell immunity. The in vitro blastogenic response of lymphocytes was studied by the MLC and the PHA tests. The incorporation rate of [^{3}H]thymidine of the T lymphocytes is given in counts per minute ± the standard error.

Subjects	MLC		PHA	
	No. tested	[^{3}H]Thymidine incorporated (count/min)	No. tested	[^{3}H]Thymidine incorporated (count/min)
Normal controls	81	26400 ± 200	81	23250 ± 210
Cancer patients				
Primary tumors	16	14894 ± 792	16	17501 ± 124
Regional spread	23	15816 ± 420	23	13345 ± 540
Distant spread	21	8968 ± 459	21	10516 ± 580
Uremic patients	26	12001 ± 272		
Transplant patients*	24	12307 ± 357		
Marihuana smokers†	34	15679 ± 499	51	13779 ± 169

* After 1 to 4 years of immunosuppressive therapy. † At least 1 year, at least once a week; no other drug taken.

such as that observed by others (*11*), and an increase in the prevalence of micronuclei. Since it has been shown that lymphocytes of normal individuals will undergo three or four divisional cycles during 72 hours of culture (*12*), the observed micronuclei might indicate that there is an increased anaphase lag with or without chromosomal breakage during the preceding cell divisions in vitro. Anaphase lag, formation of hypodiploid cells, and alterations of DNA content were also observed in cultures of human lung explants exposed to marihuana smoke (*13*). Tetrahydrocannabinol in 3 to 9 μM concentration inhibits the growth of tetrahymena by reducing DNA and RNA synthesis (*14*).

Further studies are required to elucidate the exact mechanism by which marihuana products might affect DNA synthesis and the genetic equilibrium of T (thymus derived) lymphocyte population.

GABRIEL G. NAHAS
NICOLE SUCIU-FOCA
JEAN-PIERRE ARMAND
AKIRA MORISHIMA

Departments of Anesthesiology, Surgery, and Pediatrics, College of Physicians & Surgeons of Columbia University, New York 10032

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Δ^8- and Δ^9-Tetrahydrocannabinol: Effects on Cultured Human Leucocytes

RICHARD L. NEU, M.S., HAROLD O. POWERS, Ph.D., SADDIE KING, B.A., and LYTT I. GARDNER, M.D. Syracuse, N.Y.

Almost nothing is known about the potentially harmful genetic effects of marijuana in man, although the possibility exists that it is teratogenic.[1,2] The drug has been used by man for centuries. Recent studies have established that the tetrahydrocannabinols (referred to hereafter as THC) are the active principles that affect the human central nervous system in the resin of *Cannabis sativa* L.[3,4,5] Only the optically active levorotatory forms of Δ^8- and Δ^9-THC are found in natural marijuana.[4] Total syntheses of both compounds have been reported.[6,7]

It has been reported that the hallucinogenic compound lysergic acid diethylamide (LSD) causes chromosome breaks and rearrangements in human chromosomes in cells cultured *in vitro*.[8] However, a recent well-controlled experiment has produced no definite evidence that LSD damages human lymphocyte chromosomes *in vivo* as studied from 72-hour cultures.[9]

We made the present study to determine if Δ^8-THC would cause similar chromosome aberrations when added to human blood cultures. Whole blood micromethod cultures were made from five healthy individuals using Chromosome Medium 1A (GIBCO, Grand Island, N.Y.). The cultures were incubated for 72 hours at 37°C. Twenty-four hours prior to the harvesting of the cells, Δ^8-THC was added to the cultures at concentrations of 30, 35, 40, and 45 μg/ml of medium. Absolute ethyl alcohol was used as the solvent for the THC. Earlier experiments had demonstrated that certain amounts of absolute ethyl alcohol were compatible with successful cultures. We added up to 0.01 ml of alcohol per ml of medium to whole blood cultures. The mitotic index decreased with increasing amounts of alcohol, but never went below 2.7%. When these "ethanol control" cultures were compared with cultures containing no ethanol, there was no observed difference in chromosomal morphology. Control cultures were set up containing the same amount of ethanol as was present in each of the THC cultures. Preliminary studies revealed no metaphases in cultures containing 50 μg or more of Δ^8-THC per ml of medium. Less than thirty metaphases were available for analysis in cultures with the higher Δ^8-THC concentrations because of a lower mitotic index and poorer quality meta-

From the Genetic and Endocrine Unit, Department of Pediatrics, State University of New York, Upstate Medical Center, Syracuse, N.Y.

Harold O. Powers, Ph.D. was a postdoctoral trainee of the National Institute of Arthritis and Metabolic Diseases on sabbatical leave from the State University of New York at Oswego. He has returned to his position as Professor of Biology and Genetics.

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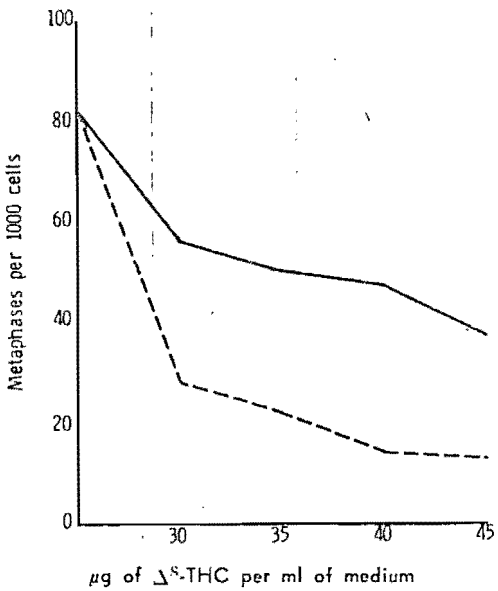

Fig. 1. A graph showing the average number of metaphase figures observed per 1000 cells at various concentrations of Δ^8-THC. The "ethanol control" cultures contained the same amount of ethanol used in each Δ^8-THC culture. Ethanol control cultures are indicated with a solid line and the Δ^8-THC cultures with a broken line.

phases. The presence of Δ^8-THC in the cultures resulted in a drastic decrease in the mitotic index at all concentrations as shown in Fig. 1. Although a decrease in mitotic index is also obvious in control cultures containing only ethanol, it is not so severe a decrease as occurs when ethanol and Δ^8-THC are present in the cultures together. No more than 5% of the metaphases in the control cultures and in those cultures exposed to Δ^8-THC had gaps and breaks. No structural changes other than these were observed. Preliminary studies with Δ^9-THC have given the same results.

Professionals in the health field are disturbed about the lack of knowledge concerning the immediate and long-term effects of marijuana. The results of this experiment indicate that, in the concentrations used, Δ^8-THC does not cause visible damage to human lymphocyte chromosomes *in vitro*. We cannot extrapolate with any degree of certainty the *in vitro* results to *in vivo* effects of THC in man, even though our negative results imply no major cytogenetic effect, since there was no observable chromosomal change. Obviously there is a need for further research with this controversial substance, especially in its relationship to the developing fetus.

Summary

Δ^8-Tetrahydrocannabinol (Δ^8-THC) was added to human leucocyte cultures to give final concentrations of 30, 35, 40 and 45 μg per ml of medium. There was a decrease in the mitotic index with increasing concentrations of Δ^8-THC. No structural rearrangements were observed. Δ^8-THC did not increase the number of breaks and/or gaps over those found in control cultures (<5%). In preliminary experiments Δ^9-THC has shown similar findings.

Acknowledgment

The (-)-trans-Δ^8-tetrahydrocannabinol used in this study was supplied by the National Institute of Mental Health through the courtesy of Dr. John A. Scigliano (batch ADL-15886-17,99.0±0.2%Δ^8-THC). The Δ^9-THC was obtained from the same source (batch QCD-61133).

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Intact humoral and cell-mediated immunity in chronic marijuana smoking

Gary S. Rachelefsky, M.D., Gerhard Opelz, M.D., M. Ray Mickey, Ph.D., Phyllis Lessin, B.A., Masahiro Kiuchi, M.D., Melvin J. Silverstein, M.D., and E. Richard Stiehm, M.D. *Los Angeles, Calif.*

The immune system of 12 healthy chronic marijuana-smoking adults was evaluated while they smoked marijuana daily for 64 consecutive days under controlled hospitalized conditions. Studies included enumeration of B and T cell subpopulations, lymphocyte proliferative responses to PHA and to allogeneic cells, and serum immunoglobulin levels. Percent B cells, initially low in 2 patients, became normal. Baseline total B cells, determined either by surface immunoglobulins (338 cells/mm³ ± 60 SEM) or complement receptors (162 cells/mm³ ± 27) were significantly ($p < 0.05$) less than control but increased to normal (485 ± 97 and 239 ± 47) over time. Percent T cells, initially low (< 40%) in 4 patients, became normal. Baseline T cells (951 cells/mm³ ± 70 SEM), significantly lower than controls (2,010 ± 210, $p < 0.05$), increased to normal by day 63 (1,875 ± 281). In vitro lymphocyte response to graded doses of PHA and to allogeneic cells was normal initially and did not change over time. Serum IgG (1,064 ± 33), IgA (166 ± 13), and IgM (96 ± 6) were normal. Serum IgE levels increased in 4 subjects without evidence of allergy. Short-term chronic marijuana use does not have a substantial adverse effect on B or T cells of young healthy adults.

Recent evaluation of cell-mediated immunity in chronic marijuana users has yielded contradicting results. A decreased T cell population[1] and a reduction of in vitro lymphocyte response to allogeneic cells and to phytohemagglutinin (PHA) have been reported.[2] In addition, Δ^9 tetrahydrocannabinol (THC), the psychoactive compound of marijuana,[3] has been reported to inhibit the PHA response of normal human lymphocytes.[4] In contrast, others have reported that chronic marijuana users had a normal in vitro response to PHA and to pokeweed mitogen[5] and normal response to skin testing with 2,4-dinitrochlorobenzene (DNCB).[6] Antibody-mediated immunity has not been adequately evaluated; low numbers of B lymphocytes have been reported.[4]

All of these prior evaluations in humans were performed on outpatient populations of chronic marijuana users. In order to better define the immunologic effects of marijuana, we studied both humoral and cell-mediated immunity in 12

From the Departments of Pediatrics, Surgery, Biomathematics, and Psychiatry, Center for the Health Sciences, University of California, Los Angeles.

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Reprint requests to: E. Richard Stiehm, M.D., Department of Pediatrics, UCLA Medical Center, Los Angeles, Calif. 90024.

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healthy young adults who smoked marijuana daily for 64 consecutive days under closely observed conditions.

MATERIALS AND METHODS

Twelve normal healthy males between the ages 21 and 28 were studied. During the 94-day research period, they lived in the UCLA Neuropsychiatric Institute under 24-hr supervision and observation by trained drug research staff to assure that the subjects did not receive any drugs other than the controlled marijuana.

All subjects were screened to obtain healthy individuals who were not regular users of drugs other than marijuana. The subjects were selected on the basis that they had not used any other drugs more than 2 to 3 times, consumed only 5 ounces of hard liquor maximum per week, no more than 3 glasses of wine or beer per day, and no more than 10 cigarettes per day over the preceding 6 mo.

Six of the 12 subjects were moderate marijuana users (3 to 6 times per week for at least the last 6 mo), and 6 were heavy users (smoked marijuana at least once daily for the preceding 6 mo). All subjects had negative medical histories and completely normal physical examinations. Baseline and monthly serial complete blood counts, urinalyses, blood chemistries, chest x-rays, and electrocardiograms were all normal.

The marijuana cigarettes were pre-rolled by a standard National Institute of Drug Abuse machine, and each contained 900 mg of marijuana containing 2.2% tetrahydrocannabinol. The subjects were required to smoke a minimum of one marijuana cigarette per day; optional cigarettes could be smoked during an 8-hr period each afternoon and evening.

After a 12-day nonintoxicated baseline period, the subjects smoked for 64 consecutive days, followed by 1 wk of nonsmoking, with resumption for 9 days and then discharge 3 days later. The mean number of marijuana cigarettes smoked daily was 5.4 ± 1.5 (SD).

Blood was drawn on each subject for in vitro immunologic studies between 7:30 and 8:30 A.M. on day 9 for baseline analysis and subsequently 25, 55, 63, and 70 days after commencing marijuana smoking, with the last test day having followed a 6-day nonsmoking period.

Normal age-matched controls consisted of healthy individuals who had never smoked marijuana. They were tested simultaneously with the marijuana smokers over the same time interval but were not kept in the hospital.

Peripheral blood lymphocytes were isolated from heparinized venous blood by Ficoll-hypaque gradient centrifugation.[7] No more than 5% of the isolated cells were granulocytes. PHA stimulation of isolated lymphocytes was performed by modification of the semimicro method of Sengar and Terasaki[8]: PHA (PHA-M, Difco Laboratories, Detroit, Mich.) in 7 titration steps (background and 100, 25, 12.5, 6.3, 3.2, and 1.6 μg PHA) was added to triplicate aliquots of 10^5 lymphocytes in 0.1-ml culture medium. At least one normal control was done simultaneously with each patient.

One-way mixed leukocyte cultures (MLC) were performed by the semimicro method described,[8] using irradiated (2,800 r) cells as stimulators. Each subject was tested as both a responder and a stimulator against 2 controls in a checkerboard fashion. One of the control cells had been frozen in liquid nitrogen and was used each time as a standard control against each marijuana subject. The variable control consisted of a different age-matched normal at each testing.

White blood counts and differentials were determined and total lymphocyte counts were calculated. T cell enumeration utilized the number of lymphocytes forming rosettes with sheep erythrocytes (E rosettes).[9] Lymphocytes surrounded by at least 3 red blood cells were considered T cells. Normal values for T cells are 56% ± 2 (SEM) and 2,010 cells/mm^3 ± 250 (SEM).

B lymphocytes were determined[10] by the presence of surface membrane immunoglobulins (Ig B cells) and by lymphocyte complement receptors (EAC rosettes).[11]

Normal Ig B cells was 16% ± 2 (SEM) with a total 520 cells/mm^3 ± 60 (SEM), while normal EAC rosettes was 9.9% ± 3 (SEM) and 285 cells/mm^3 ± 48 (SEM).

Serum levels of IgG, IgM, and IgA immunoglobulins were measured by single radial dif-

TABLE I. Change in T and B lymphocytes over time in 12 chronic marijuana smokers compared to normal subjects

Immunologic test	Baseline (Mean ± SEM)	Representative days post-smoking (Mean ± SEM)		Pooled values (Mean ± SEM)	Normal (Mean ± SEM)
		25	63		
T cells*					
Percent	48 3	45 4	53 3	48 4	56 2
Total (cells/mm³)	951 70†	1,300 183†	1,875 281	1,330 78†	2,010 210
Number tested (n)	n = 12	n = 10	n = 10	n = 54	n = 56
B cells					
A. Ig-stain‡					
Percent	18 2	17 3	18 2	17 2	16 2
Total (cells/mm³)	338 60†	469 101	485 97	434 35	520 60
Number tested	n = 12	n = 10	n = 10	n = 53	n = 52
B. EAC rosettes§					
Percent	8 4	8 6	6 2	8 4	10 3
Total (cells/mm³)	162 27†	160 50‖	239 47	206 21†	285 48
Number tested	n = 12	n = 9	n = 10	n = 51	n = 52

*Determined by 3 or more lymphocyte-forming rosettes with sheep erythrocytes.[9]
†Less than normal, $p < 0.05$.
‡Detected by immunofluorescent staining of surface membrane immunoglobulins.[10]
§Detected by lymphocyte receptors.[11]
‖Less than normal, $p < 0.08$.

fusion using immunoglobulin standards certified by the World Health Organization.[12] Serum IgE levels were measured with Phadebas IgE test kit. Mean normal adult value was 70 international units (IU)/ml ± 28 (SEM).

Results were summarized using computer programs P3D and P7D.[13] Averages and standard deviations were computed for each variable for each time period separately, for each subject averaged over time, and for all readings combined into a single data pool. The latter, for purpose of analysis, will be referred to as pooled results. In the case of cell counts, phytohemagglutinin (PHA) and MLC stimulation counts, and IgG, IgM, IgA, and IgE determinations, data were evaluated on both the arithmetic and logarithmic scale; statistical comparisons were based on the latter.

RESULTS

Results of immunologic tests were not influenced by number of cigarettes each subject had smoked per day before or during the study period.

Table I depicts the T and B lymphocyte results over time. The mean percent T cells remained within normal range during the study period. Absolute T cells were decreased initially and increased significantly ($p < 0.01$) to a normal level (951 cells/mm³ ± 70 SEM to 1,875 ± 281) from baseline to day 63.

T cell percent was initially depressed (< 40%) in 4 (of 12) subjects, with 2 becoming normal by 25 days and 2 by 55 days postmarijuana smoking.

Pooled B cell determinations (Ig B cells 17% ± 2 SEM; EAC rosettes 8% ± 4) were not significantly different from the normal group. Initially, 2 (of 12) subjects had depressed (< 8%) B lymphocytes (as determined by immunoglobulin staining), with both becoming normal by day 55. One patient experienced a depression (27 to 7%) by day 63. One other patient had a decrease in EAC rosettes (8 to 1% by day 25) and remained low. Absolute number of B lymphocytes by either method was significantly ($p < 0.05$) lower than normal initially

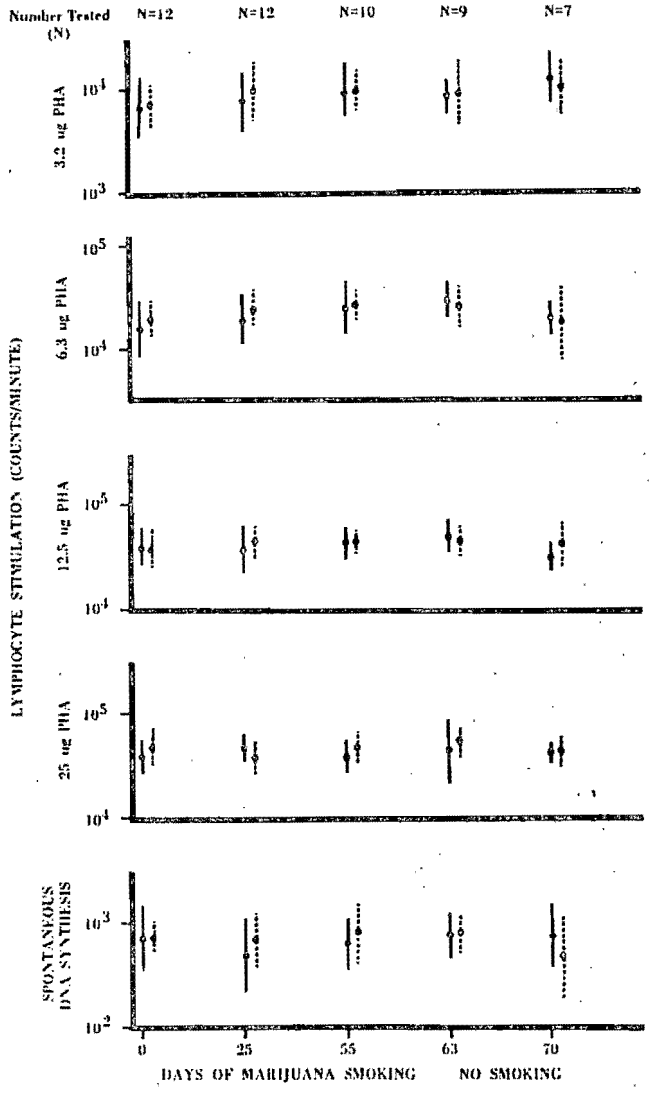

FIG. 1. Representative peripheral blood lymphocyte stimulation[8] by graded dose of phytohemagglutinin (PHA—25, 12.5, 6.3, and 3.2 μg) in 12 subjects during 63 days of marijuana smoking and after the 6-day nonsmoking period compared to controls evaluated simultaneously. ┼ represents marijuana subjects' response (cpm) ± 1 SD; ┆ represents controls response ± 1 SD.

but increased to normal by day 25 for Ig B cells and by day 63 for EAC rosettes.

In vitro lymphocyte response to PHA was normal at all dilutions of PHA in each patient and in the combined group initially and remained such without a significant change during the smoking and nonsmoking periods (Fig. 1). The pooled responses at the background (subjects 705 counts per minute [cpm] ± 81 SEM vs controls 724 ± 66) and at all PHA concentrations were normal (i.e., for 100 μg, 25 μg, 12.5 μg, 6.3 μg, 3.2 μg, and 1.6 μg PHA concentrations the subjects'

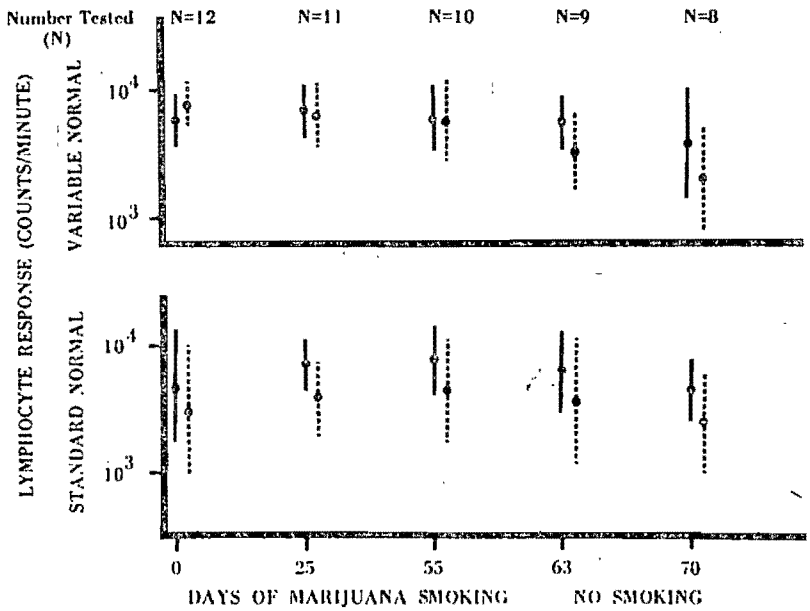

FIG. 2. Mixed leukocyte culture response[8] of peripheral blood lymphocytes obtained from 12 subjects during 63 days of marijuana smoking and after the 6-day nonsmoking period compared to a variable and standard control. The latters' lymphocytes were obtained at the beginning of study and frozen in liquid nitrogen and tested each time. Each subject was tested as both a responder and a stimulator against the 2 controls in a checkerboard fashion. ⟊ is subjects' lymphocyte response to control (cpm) ± 1 SD; ⟊ is controls' lymphocyte response to marijuana subjects' ± 1 SD.

cpm $1 \times 10^2 \pm 1$ SEM were 488 ± 23, 479 ± 24, 397 ± 25, 225 ± 17, 87 ± 8, and 32 ± 3 compared to 519 ± 34, 509 ± 24, 442 ± 23, 250 ± 17, 97 ± 8, and 32 ± 6 in the controls, respectively).

The marijuana subjects' MLC response (Fig. 2) to the standard and variable control was normal initially (4,909 cpm ± 781 SEM and 6,081 ± 450) and did not change significantly individually or as a group over time. The stimulatory ability of the marijuana smokers' lymphocytes was lower than its corresponding response to either control.

Serum IgG (mean total group 1,064 mg/100 ml ± 33 SEM), IgA (166 ± 13), and IgM (96 ± 6) were normal in all patients compared to age-matched controls.[12] There were no significant changes over time. Initial serum IgE level was elevated (1,200 IU/ml) in 1 patient, though the geometric mean for the 11 subjects tested (57 IU/ml ± 11 SEM) was normal. The IgE levels increased in 4 (of 8) patients by day 55, with the levels increasing from 33 IU/ml to 105, 18 to 75, 32 to 244, and 59 to 123. Allergic manifestations (i.e., urticaria, wheezing, etc.) were not observed.

DISCUSSION

Our findings appear to dispute other reports describing an association between chronic marijuana smoking and decreased humoral and cell-mediated immunity.

No depression of the in vitro response to PHA or allogeneic cells was observed in our population of chronic marijuana users. Initially depressed T cells and B cells became normal in spite of marijuana use.

B lymphocyte numbers returned to normal in those patients with initial depressed levels, with 2 patients demonstrating a decrease over time. Levels of IgA, IgG, and IgM were unaffected. IgE levels increased with time, suggesting the development of specific IgE, though none of our patients experienced allergic reactions. IgE-mediated sensitization to marijuana has been described.[11]

Nahas and associates,[2] in evaluating 51 chronic marijuana smokers, reported depressed incorporation rates of ^{3}H-thymidine by lymphocytes in response to PHA and to allogeneic lymphocytes (in 34 patients). The level of depression was comparable to that observed in advanced cancer and uremic patients, both groups with known impairment of T cell function. In a separate study, Nahas and associates[1] reported that 1.6 to 20 μg of THC inhibited the PHA response of normal human lymphocytes when added to in vitro cultures. In contrast, White, Brin, and Janicki,[5] in evaluation of 12 healthy chronic marijuana smokers, observed no significant difference of in vitro lymphocyte response to PHA or to pokeweed mitogen when compared to 12 age-matched controls.

The preceding stimulation studies used a single dose of PHA; a dose response to PHA as in our study was not evaluated. The results obtained by us revealed no abnormality in response of peripheral blood lymphocytes to any concentration of PHA. Response to allogeneic lymphocytes was also normal and unaffected by marijuana use.

In one study,[1] T lymphocytes (%) were decreased in 9 of 23 marijuana users, with the overall group having a significant decrease compared to 23 controls. Six of the 9 patients remained with low T cells on repeat 14 days later. Total T lymphocytes were not reported. We confirm low T cells in such a group of young adults. However, our data suggest that it is not "pure" marijuana but other factors that account for this observation.

In vivo cellular immune function in marijuana users has been reported to be normal. Silverstein and Lessin[6] performed skin testing to 2,4-dinitrochlorobenzene in 22 subjects and observed that all had the capacity to become sensitized and develop cutaneous hypersensitivity to this agent while 20% (12/60) untreated cancer patients had negative responses. It has been noted that DNCB will sensitize 80% to 95% of a normal population.[15] Twenty (of the 22) had positive delayed-type hypersensitivity reactions to at least 1 of 4 common skin test antigens, while Gupta, Grieco, and Cushman[3] observed that 5 (of 16) of his subjects were unable to manifest positive reactions, with a normal expected response rate being 90%.[16] In the latter study, the presence or absence of anergy was not related to percent T cells, i.e., 3 of 8 with low T cells and 2 of 8 with normal T cells had negative skin tests.

In the only B lymphocyte evaluation in chronic marijuana smokers, Gupta, Grieco, and Cushman[3] (measuring EAC rosettes) observed that their 23 patients had normal percentage (11.6% ± SD) though 5 subjects had "low" values (no values were given). Total B cells were not reported. Serum immunoglobulins have

not been reported in chronic marijuana smokers, though IgM is elevated in heroin addicts.[17]

The different effects of marijuana or its active component on lymphocyte in vitro and in vivo function observed to date are difficult to reconcile. Prior studies have involved a mixture of poorly defined populations lumped together as chronic marijuana smokers with each study evaluating one aspect of the immune system. Depressed B and T lymphocytes reported previously and observed initially in our population might be a result of impurities or other chemicals incorporated into "street" marijuana. None of the previous authors could be sure that their test subjects were not using any other medications or illicit drugs. Even the ingestion of aspirin can inhibit the in vitro reactivity of lymphocytes.[18] A recent viral infection could also influence lymphocyte function.[19] None of our patients were ill, and no other drugs were administered during our study period. Thus, we were provided with a well-controlled population of chronic marijuana smokers. No substantial adverse effect on B or T lymphocytes or on in vitro PHA or MLC response was demonstrated.

ADDENDUM

Since preparation of the manuscript, Lau and associates* reported results comparable to ours in a study of the effects of oral THC. Eight healthy chronic marijuana smokers were hospitalized for 30 days. Each subject received a capsule containing either placebo or THC. A sustained dose of 210 mg THC per day was given for 18 days. PHA response of their lymphocytes was normal at baseline and was unaffected by the ingestion of THC.

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INHIBITION OF GLUCOSE EFFLUX FROM HUMAN ERYTHROCYTES BY HASHISH COMPONENTS

AVITAL SCHURR, NINA SHEFFER, YOSEF GRAZIANI and AVINOAM LIVNE

Department of Biology and Research and Development Authority, University of the Negev, Beer Sheva, Israel

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Abstract—Glucose efflux from human erythrocytes under zero-*trans* conditions is inhibited by the hashish components Δ^1-tetrahydrocannabinol and cannabidiol at concentrations above 5×10^{-7} M. The inhibitory effect is rapid and apparently readily reversible. Increasing concentrations of alcohols up to 20 mM do not change the rate of glucose efflux but they do amplify the extent of efflux inhibition by a given dose of hashish component. Methanol, ethanol and propanol show this amplifying effect in a decreasing order.

"MARIHUANA HUNGER", the special craving for sweets, is a common symptom of marihuana smoking. This symptom may indicate a lowered blood glucose level, however, on the basis of glucose tolerance test, Podolsky *et al.*[1] have shown no lowering of blood glucose level. Rather, these authors detected hyperglycemic changes attributable to the drug.

The effects of the hashish compounds, Δ^1-tetrahydrocannabinol (Δ^1-THC) and cannabidiol (CAN) on rat and human erythrocytes[2,3] and on rat liver mitochondria[4,5] and lysosomes[6] have been described recently. A paramount feature of the effects is an interaction between the hashish compounds and the membrane of these cells or organelles. It seems therefore possible that the hyperglycemic changes might be related, among other factors, to an effect of the hashish compounds on glucose transport across cellular membranes.

As an approach to study such an effect in a defined system, we examined glucose transport in human erythrocytes. This paper illustrates a marked effect of the hashish compounds on glucose transport, an effect which is accentuated by added alcohols.

MATERIALS AND METHODS

Freshly drawn human blood in heparin or stored transfusion blood (up to 3 weeks of storage) were used in this study, showing essentially the same results. The procedure for washing the erythrocytes and for measuring glucose under zero-*trans* conditions was as described by Karlish *et al.*[7] Two modifications of this procedure were introduced. First, 0·4 ml of the cell suspension (60% hematocrit) preloaded with 80 mM glucose, was rapidly mixed with 100 ml of the zero-*trans* washout medium. Secondly, glucose analysis was performed in part of the experiments with glucose oxidase.[8] In these experiments, glucose was determined by the use of a colourimetric assay in which production of a coloured dye formed from *o*-dianizidine in a peroxide coupled reaction was measured. Parallel experiments verified that the results of this

analysis were identical to those obtained with radioactive glucose. The glucose oxidase reaction products and the hemoglobin content, using standard Drabkin procedure,[9] were determined colourimetrically, using Klett Summerson colorimeter with 42 and 54 filters, respectively.

All chemicals were of analytical grade. Δ^1-THC and CAN were kindly supplied by Prof. R. Mechoulam (School of Pharmacy, The Hebrew University, Jerusalem). The hashish compounds were introduced as alcoholic solutions and the control systems contained the same amount of alcohol.

RESULTS

Glucose transport across the human erythrocyte membrane has been studied by exchange[10,11] and by net flux measurements, either according to Sen-Widdas,[12] or according to the zero-*trans* procedure of Stein and co-workers.[7,13] The exchange and the Sen-Widdas net flux measurements require the presence of glucose on both sides of the membrane. Such requirement might complicate the interpretation of the effect of the hashish components. The zero-*trans* procedure, on the other hand, maintains the concentration of glucose at the *trans* (outer) face of the membrane at or near zero.[7] This procedure was therefore adopted for the present study.

Glucose efflux from human erythrocytes was inhibited by Δ'-THC and CAN at concentrations above 5×10^{-7} M. Representative data, depicting the effect of Δ'-THC and CAN on glucose efflux are shown in Fig. 1. The ordinate f values are the ratio of amount of glucose, present in the cells at any time, related to its amount in the cells at time zero.[7] CAN is significantly more effective than Δ'-THC in arresting glucose efflux. Table 1 presents the kinetic parameters[7] of the efflux data given in Fig. 1. While the K_m value increases significantly with increasing concentrations of the hashish components, V_{max} is only slightly affected by these components.

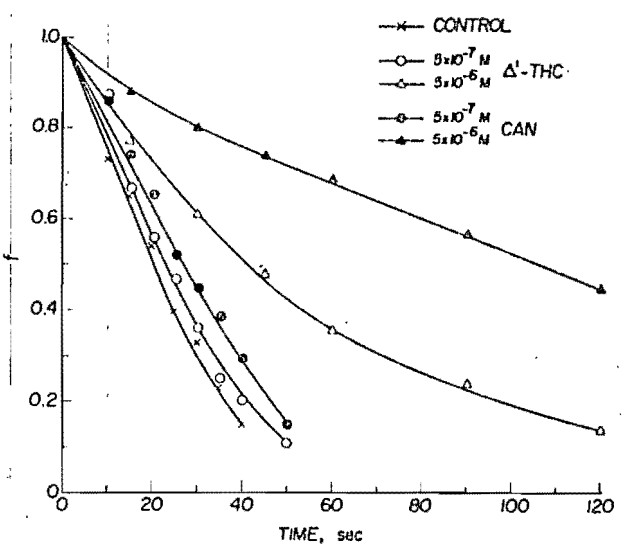

FIG. 1. Effect of Δ'-THC and CAN on glucose efflux from human erythrocytes. The ordinate f values are the ratio of amount of glucose, present in the cells at any time, related to its amount in the cells at time zero.[7]

TABLE 1. GLUCOSE EFFLUX FROM HUMAN ERYTHROCYTES UNDER ZERO-*trans* CONDITIONS AS AFFECTED BY HASHISH COMPONENTS

Addition (M)	n	K_m (mM)	V_{max} (μmoles/min per l. isotonic cell water)
Control	13	22 ± 2	189 ± 19
Δ'-THC			
5×10^{-7}	6	35 ± 3	198 ± 18
5×10^{-6}	6	63 ± 15	208 ± 29
CAN			
5×10^{-7}	6	43 ± 4	216 ± 21
5×10^{-6}	6	158 ± 30	217 ± 21

The cannabinoids were added from methanolic stock solutions; final methanol concentration in reaction mixtures: 32 mM. n = number of experiments. K_m and V_{max} were calculated according to Karlish *et al.*[7] Mean values ± S.E. are given.

Glucose loaded erythrocytes (60 per cent cell suspension) were incubated with 10^{-5} M CAN for 5 min at 37° and then tested for glucose efflux by mixing an aliquot with the washout medium (250 fold dilution). Under these conditions CAN caused no change in glucose efflux, in contrast to its profound inhibitory effect when added directly to the efflux medium, as shown in Fig. 1. Apparently the effect of the hashish compounds is readily reversible, as already shown with respect to erythrocyte stabilizing effect of these compounds.[3]

Since Δ^1-THC and CAN are not water soluble, they were added from methanolic or ethanolic stock solutions. While the alcohols, at a concentration range of

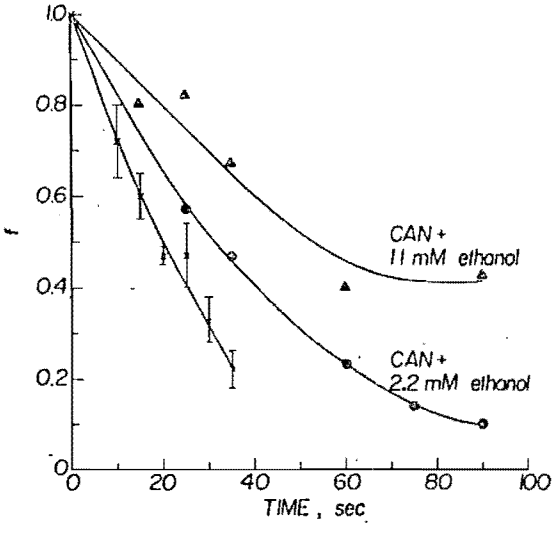

FIG. 2. Effect of ethanol concentration on the inhibition of glucose efflux by 5×10^{-6} M CAN. Standard errors for the control, including the ethanol treatments without CAN, are given as vertical bars. The ordinate *f* values are as in Fig. 1.

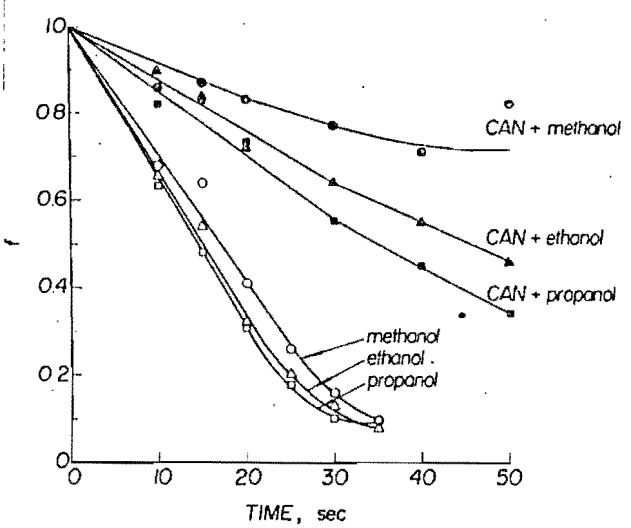

FIG. 3. Effect of 12·5 mM alcohol on the inhibition of glucose efflux by 5×10^{-6} M CAN. The ordinate f values are as in Fig. 1.

2×10^{-3} to 2×10^{-2} M barley changed glucose efflux, they did, however, amplify the inhibition of glucose efflux by the hashish components. Figure 2 shows that the extent of efflux inhibition by a given dose of CAN is increased with increasing alcohol concentration. The increased inhibition due to alcohol is significant. Furthermore, Fig. 3 shows that this amplifying effect depends on the length of the alcohol aliphatic chain: methanol, ethanol and propanol are effective in a decreasing order.[4] This trend appeared in all three experiments.

DISCUSSION

Various membrane systems are affected by Δ^1-THC and CAN.[2–6] These reports along with the present study, indicate that cellular membranes are indeed a major site of interaction with the hashish components. The effect of Δ^1-THC and CAN on both osmotic fragility[3] and glucose efflux are very rapid and readily reversible, but while Δ^1-THC is more effective than CAN with respect to conferal of osmotic stability,[3] the opposite is true with regards to inhibition of glucose efflux (Fig. 1). This disparity and the multiplicity of membrane systems and functions affected by hashish components exclude the possibility of a single site and mechanism for the various effects of the hashish components.

The kinetic properties of the drug-affected efflux (Table 1) show features of competitive inhibition, as already demonstrated for phloretin.[14] If the inhibition is indeed competitive, it is expected that it will be dependent on the erythrocyte glucose concentration. However, the degree of inhibition of glucose efflux by 5×10^{-6} M CAN was independent of glucose concentration at a range of 30–120 mM. In view of the interaction of the cannabinoids with phospholipids[4] and with lipoproteins,[15] it is possible that these drugs modify the lipophylic environment of the glucose carrier[11,16] and thus change the apparent affinity of the carrier to glucose.

The physiological significance of the inhibition of glucose transport by Δ^1-THC and CAN should be considered. For hashish smokers, temporary halt in glucose

transport from the plasma into the erythrocytes is possible, particularly since the effective concentration of the drugs in inhibiting glucose transport (Fig. 1) correlates with the doses leading to physiological reactions in hashish smokers.[17,18] It is possible that the hashish components affect glucose transport not only in erythrocytes but in other cells as well, thus leading eventually to side-effects and symptoms of hashish smoking, such as "marihuana hunger", hyperglycemia and glucosuria. It is noteworthy that several lines of evidence indicate that glucose transport into adipose cells[19] and across the muscle cell membrane[20] show distinct properties of facilitated diffusion which are also typical for human erythrocytes.

The interacting effects of alcohols and the hashish components in inhibiting glucose transport (Figs. 2 and 3) may be due to an increase of the effective drug concentration in the membrane by the alcohol. Interestingly, hashish smokers have disclosed to us a custom of drinking alcoholic beverages while smoking hashish in order to attain a more pronounced "high". This claim of an *in vivo* interaction of alcohol and hashish should be rigorously tested.

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Normal Skin Test Responses in Chronic Marijuana Users

Abstract. *The cell-mediated immunity of 22 chronic marijuana smokers showed no difference from that of normal controls when evaluated by in vivo skin testing with 2,4-dinitrochlorobenzene. However, a significant difference was seen between these chronic marijuana users, all of whom could be sensitized to 2,4-dinitrochlorobenzene, and age-matched cancer patients, who showed a decreased capacity to be sensitized.*

In a study of 51 subjects, Nahas (*1*) suggested that cellular immunity may be impaired by the chronic use of marijuana. His in vitro studies showed that lymphocyte blastogenesis in response to allogeneic cells (mixed lymphocyte culture) and in response to the mitogen phytohemagglutinin was decreased to levels comparable to those of patients with a known impairment of cellular immunity (cancer, uremia, and kidney transplant patients), and that this depression was statistically different from that of normal controls. However, in vivo skin testing with the foreign antigen 2,4-dinitrochlorobenzene (DNCB) and with a battery of common antigens may be a better gauge of overall immunocompetence than any currently used in vitro lymphocyte function test, because skin testing closely correlates to clinical prognosis in cancer patients (*2*). Since 96 percent of the normal population can be sensitized to DNCB (*2*, *3*), we tested the capacity of chronic marijuana users to develop a delayed cutaneous hypersensitivity response to DNCB and thus grossly evaluated, in vivo, their immunologic competence.

For this study, the chronic marijuana user was defined as one who regularly smoked marijuana a minimum of three times per week for at least 6 months (*4*). Tobacco smoking and alcohol were allowed but the regular or frequent use of other drugs disqualified the subject from the study. Skin testing with DNCB was performed in a standard fashion (*2*) on 22 healthy males ranging in age from 21 to 30 years. After the skin was cleansed with acetone, a sensitizing dose of 2000 μg of DNCB dissolved in 0.1 ml of acetone was applied to the skin of the upper arm. A 100-μg dose was applied at the same time to the ipsilateral forearm to test for previous sensitization. After 14 days, the subjects were challenged on the ipsilateral forearm with 100, 50, and 25 μg of DNCB as well as with a cutaneous irritant, croton oil, as a check for an intact inflammatory response. On the same day, four common antigens in doses of 0.1 ml were injected intradermally in the contralateral forearm. The antigens were monilia (Hollister-Stier, 2 units), mumps (Lilly, 2 units), purified protein derivative (Connaught, 5 units, intermediate strength), and Varidase (Lederle, 10 units).

All positive reactions were scored as 1+ (erythema and induration of less than half the diameter of the test site), 2+ (measurable skin reaction over at least half of the test site), 3+ (reaction covering the entire test site), or 4+ (bullae formation).

All 22 subjects reacted to 50 μg of DNCB (21 were either 3+ or 4+), and 21 reacted to 25 μg of DNCB (14 were either 3+ or 4+). Seventeen subjects reacted to two or more common antigens, three subjects reacted to only one common antigen, and two subjects failed to react to any of them.

The failure of two DNCB-positive subjects to respond to any of the common antigens is probably not indicative of a defect in immunologic memory. In this age group, unresponsiveness is probably due to lack of exposure to these antigens.

The results of the DNCB skin testing are summarized in Table 1 and are compared with combined data from published evaluations of DNCB skin testing in normal subjects and cancer patients (*2*, *3*). In order to obtain an age-matched control group with probable immunodepression, we reviewed the skin test records of 60 consecutive cancer patients between the ages of 21 and 30 years from the Division of Oncology, University of California, Los Angeles. These patients, who were not under drug treatment at the time of testing, were tested by the identical procedure in the same institution as the marijuana users and represent a concurrent control group. Twelve (20 percent) were DNCB-negative. Thus, this DNCB procedure can detect a defective immune response.

When our group of 22 DNCB-positive, chronic marijuana users was compared to the control group of 279 normal subjects, there was no statistical difference (*5*, *6*). It appears that the chronic use of marijuana does not decrease the capacity of a subject to become sensitized and to develop delayed cutaneous hypersensitivity when challenged with the hydrocarbon hapten DNCB. However, when the marijuana smokers were compared with either cancer group [all ages (*5*, *7*) or ages 21 to 30 (*5*, *8*)], a statistical difference was found.

The 10 percent difference in DNCB positivity between all cancer patients and the age-matched cancer patients, although not significant (*5*, *9*), may be due to the fact that many patients in the age-matched group had early, localized disease. Immunocompetence appears to decrease with increasing age and extent of neoplastic disease.

Prolonged immunosuppression has profound implications. Patients with congenital immunodeficiencies and those with iatrogenic immunosuppression (such as renal transplant patients) develop cancer at rates at least 80 times that of the general population (*10*). However, there is no clinical or epidemiologic evidence to suggest that chronic marijuana users might be more prone to the development of neoplastic or infectious processes.

In vivo skin testing has proved to be a valuable tool in the gross evaluation of the immune system because a delayed cutaneous hypersensitivity response requires the participation of a number of components of cell-mediated immunity. Since responses were normal in the chronic marijuana users we tested, it would appear that chronic

Table 1. 2,4-Dinitrochlorobenzene reactivity.

Groups	Subjects (No.)	DNCB-positive		DNCB-negative	
		Number	Percent	Number	Percent
Marijuana smokers	22	22	100	0	0
Normal controls*	279	267	96	12	4
Cancer patients (all ages)*	548	384	70	164	30
Cancer patients (ages 21 to 30)†	60	48	80	12	20

* Data from published studies (*2*, *3*). † Concurrent controls.

marijuana smoking does not produce a gross cellular immune defect that can be detected by skin testing. Further study is needed to evaluate chronic marijuana use and its effect, if any, on the immune system.

MELVIN J. SILVERSTEIN
PHYLLIS J. LESSIN
Department of Surgery, Division of Oncology, and Department of Psychiatry, Center for the Health Sciences, University of California,

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4. Marijuana smoking ranged from three times per week to several times per day. No marijuana was administered by us to these subjects, so the specific dosage levels or percentage of tetrahydrocannabinol of the marijuana smoked is not known.
5. Statistical analyses performed were the Yates modification of the chi-square procedure and Fisher's exact probability test (two-tailed).
6. $\chi^2 = 0.18$, $P = .669$; Fisher's exact $P = 1.000$.
7. $\chi^2 = 7.84$, $P = .005$; Fisher's exact $P = .001$.
8. $\chi^2 = 3.68$, $P = .055$; Fisher's exact $P = .030$.
9. $\chi^2 = 2.13$, $P = .144$; Fisher's exact $P = .133$.
10. J. Penn and T. E. Starzl, *Transplantation* 14

The effect of delta-9-tetrahydrocannabinol on the chromosomes of human lymphocytes in vitro

MORTON A. STENCHEVER, M.D., F.A.C.O.G.
MARJORIE ALLEN
Salt Lake City, Utah

Human leukocytes from 4 healthy donors were exposed to concentrations of delta-9-tetrahydrocannabinol in 72 hour tissue cultures. Concentrations studied were 100.0, 10.0, 1.0, and 0.1 μg per milliliter of culture material. For each experiment a control culture was evaluated. There was no increase in the incidence of chromosome breaks or gaps in any of the study cultures when compared to controls. The 100.0 μg cultures did not grow in tissue culture, and no cells in mitosis were noted in any preparations from this concentration. The significance of these negative data is discussed.

DELTA-9-tetrahydrocannabinol is the major active component in marihuana. This drug is being widely used by individuals from all strata of society. It is primarily used by young individuals who are well within their reproductive years. Persaud and Ellington[4, 5] have demonstrated that cannabis resin is teratogenic in the rat but causes only fetal resorption and stunting in the mouse. Pace and associates,[3] on the other hand, could not demonstrate teratogenic activity in the rat in a variety of experiments. While no epidemiologic outbreak of teratogenicity in human beings has been seen, it cannot be stated with assurance that the use of this agent is not teratogenic in the human being, and certainly more research needs to be carried out.

Neu and colleagues[2] studied delta-9-tetrahydrocannabinol in in vitro experiments with human leukocytes, and were able to find no increase in chromosome abnormalities. Pace and associates[3] could find no significant chromosome breakage in rat cells after exposure to marihuana. Gilmour and co-workers[1] in studying several psychoactive drugs for chromosome aberrations in vivo looked at cultures from 16 "light" users and were unable to find any significant chromosome anomalies. However, an increase in chromosome breakage was noted in 11 "heavy" users.

It is the purpose of this presentation to present data obtained from in vitro studies of the effect of delta-9-tetrahydrocannabinol on human leukocyte cells.

Material and methods

Primary leukocyte cultures were initiated from 4 healthy subjects (2 male and 2 female) ranging in age from 19 to 33 years. None were using any drugs and they had not been exposed to any agents, such as x-ray, which might be implicated in chromosome breakage. The women were not using oral contraceptives, and all 4 were free of viral illnesses at the time the cultures were initiated. Twenty milliliters of heparinized blood

From the Department of Obstetrics and Gynecology, University of Utah College of Medicine.
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Reprint requests: Morton A. Stenchever, M.D., 50 North Medical Dr., Salt Lake City, Utah 84112

Table I. Chromosome gap and break data for human leukocytes treated with delta-9-tetrahydrocannabinol

Concentration (μg/ml.)	No. of cells scored	Cells with gaps		Cells with breaks	
		No.	Per cent	No.	Per cent
Subject 1 (male)					
100.0	–	–	–	–	–
10.0	301	3	1.0	9	2.9
1.0	280	2	0.7	0	0
0.1	275	4	1.4	2	0.7
0.0*	300	5	1.6	6	2.0
Subject 2 (female)					
100.0	–	–	–	–	–
10.0	215	6	2.7	8	3.7
1.0	232	4	1.7	2	0.8
0.1	230	8	3.4	9	3.9
0.0*	203	7	3.8	4	1.9
Subject 3 (male)					
100.0	–	–	–	–	–
10.0	204	0	0	3	1.8
1.0	201	0	0	4	1.9
0.1	200	1	0.5	2	1.0
0.0*	200	1	0.5	3	1.5
Subject 4 (female)					
100.0	–	–	–	–	–
10.0	200	2	1.0	5	2.5
1.0	200	7	3.5	2	1.0
0.1	200	1	1.0	0	0
0.0*	200	9	4.5	11	5.5

*Control.

was drawn from each subject and treated with phytohemagglutinin for 30 minutes in ice. Following this, the specimen was centrifuged at 500 r.p.m. for 5 minutes. The lymphocyte-containing sera were separated into 5 equal portions in small Erlenmeyer flasks. Dulbecco's modified Eagle's medium with penicillin and streptomycin was added to each flask to make a final volume of 10 ml. One flask served as a control, and, to this, ethanol to make a final concentration of 0.1 per cent was added. Delta-9-tetrahydrocannabinol in ethanol was added to each of the other flasks to make final concentrations of 100.0, 10.0, 1.0, and 0.1 μg per milliliter of culture media. In each, the final concentration of ethanol was also 0.1 per cent. The flasks were then cultured at 37° C. for 72 hours. Two hours prior to harvesting, 0.2 mg. of Colcemid was added to each flask, and harvesting was carried out with an air-dry technique. Slides were stained with carbofuchsin and coded for blind scoring. Slides from each culture were then scored for chromatid and isochromatid breaks and gaps and for the presence of abnormal forms.

Results

Table I summarizes the data from the 4 experiments. No growth was seen in any of the 100.0 μg per milliliter concentration flasks. No mitotic figures were seen in any of these preparations. Indeed, only rare cells were seen at all, and these exhibited karyolysis and fragmentation of nuclei. No evidence for transformation of lymphocytes was noted. Although the remaining cultures grew well and demonstrated a good mitotic index, there was no significant increase in chromatid or isochromatid breaks or gaps in any of the concentrations when compared to the controls. No abnormal forms were seen in any of the cultures. For tabulation purposes, chromatid and isochromatid breaks are considered as "breaks," and chromatid and isochromatid gaps as "gaps."

Comment

These in vitro data are negative. However, an abundance of work has not been carried out in determining whether or not this agent actually breaks chromosomes. It has been our observation on sporadic cases that users seem to have an increased number of chromosome breaks when compared to other individuals studied in our laboratory. Although in vitro studies with pure delta-9-tetrahydrocannabinol did not demonstrate breaks, it is not possible to rule out other possibilities. It may be that impurities in the marihuana may cause chromosome breaks and do genetic damage. It is also possible that metabolic products of some ingredient in marihuana may be capable of causing chromosome breaks in vivo. Since there is evidence for possible teratogenicity[4, 5] and since the use of marihuana is so widespread, it seems appropriate that these possibilities be investigated in detail in future studies. Careful human observations must be made, and a variety of animal species must also be studied in well-controlled experiments. Our laboratory is currently studying a large number of youthful users for the presence of chromosome abnormalities, and an attempt is being made to correlate frequency of use with outcome. Although Gilmour and coworkers[1] were unable to find any increase in chromosome abnormalities in light users, there did seem to be an increase in 11 heavy users in their study. Unfortunately, these individuals were frequently using other drugs as well, including lyseric acid diethylamide. We are attempting to study individuals using only marihuana. While it is difficult to find such individuals, it is important that this be done in order to discover the effect of this drug alone.

Another aspect to be identified is the fact that certain individuals may be more prone to chromosome damage by specific agents than others. This information must be ascertained if possible for marihuana, as well as for other drugs, if a meaningful potential risk is to be determined. Certainly, we are only at the beginning of our investigations, and it is hoped that several investigators will continue the study of the effects of this drug on human cells and report their findings. It is as yet unknown precisely what blood levels of the drug are attained in heavy users. Subpharmacologic doses injected into volunteers by Lemberger and associates[6] apparently produced blood levels far below the higher concentrations investigated in this study.

Finally, it is important to emphasize that in vitro chromosome breakage studies must be well controlled and scored in a blind fashion to eliminate bias. Spontaneous breakage and gap rates vary from laboratory to laboratory, method to method, and, indeed, experiment to experiment. Widely varied concentrations of the agent should also be tested. In this experiment, concentrations of from 0.1 to 100.0 μg per milliliter were used, the latter being completely toxic with no growth seen. If chromosome breakage is considered a potential sign of nuclear damage, the in vitro system allows for the control of most variables. However, it must be remembered that even within this system variables exist, and data obtained from such experiments, whether positive or negative, must be interpreted as part of the total picture and not as conclusive evidence for the drug's danger or safety.

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SUBACUTE EFFECTS OF HEAVY MARIHUANA SMOKING ON PULMONARY FUNCTION IN HEALTHY MEN

DONALD P. TASHKIN, M.D., BERTRAND J. SHAPIRO, M.D., Y. ENOCH LEE, B.S., AND CHARLES E. HARPER

Abstract To evaluate the subacute effects of heavy marihuana smoking on the lung, pulmonary function was tested in 28 healthy young male experienced cannabis users, before and after 47 to 59 days of daily ad-libitum marihuana smoking (mean of 5.2 marihuana cigarettes per day per subject, 2.2 per cent Δ9-tetrahydrocannabinol). Base-line pulmonary-function studies were nearly all within normal limits, but after 47 to 59 days of heavy smoking, statistically significant decreases in forced expired volume in one second (3 ± 1 per cent, S.E.), maximal mid-expiratory flow rate (11 ± 2 per cent), plethysmographic specific airway conductance (16 ± 2 per cent) and diffusing capacity (8 ± 2 per cent) were noted as compared with the base-line studies. The decreases in maximal mid-expiratory flow rate and specific airway conductance were correlated with the quantity of marihuana smoked. These findings suggest that customary social use of marihuana may not result in detectable functional respiratory impairment in healthy young men, whereas very heavy marihuana smoking for six to eight weeks causes mild but statistically significant airway obstruction. (N Engl J Med 294: 125-129, 1976)

THE increasing frequency of marihuana use in the United States over the past decade[1] has stimulated greater interest in its possible health hazards. Since the principal route of administration in this country is by deep inhalation into the lungs, there is obvious concern regarding possible adverse effects on the respiratory tract, particularly in view of the overwhelming evidence of the harmful effects of chronic tobacco cigarette smoking on the lung. Although acute airway dilatation has been noted after smoked marihuana both in healthy young men[2,3] and in patients with bronchial asthma,[4,5] reports of bronchitis after heavy or chronic smoking of marihuana[6-8] suggest that the long-term effects on the lung may be deleterious, although these reports are either anecdotal[6] or based on observations in which the variable of tobacco smoking was not controlled.[7,8] Since tests of pulmonary function are capable of detecting abnormalities in even young, asymptomatic smokers of tobacco, suggesting probable early airways disease,[9-14] the present study was designed to determine prospectively whether or not and to what extent heavy marihuana smoking over a period of approximately seven to nine weeks might cause functional respiratory impairment in healthy young experienced cannabis users and whether the quantity of marihuana smoked over this period might be related to the degree of impairment of lung function.

MATERIAL AND METHODS

Volunteers were recruited through newspaper advertisements. Only healthy male experienced marihuana smokers were accepted for study. Of the 30 subjects selected, one withdrew shortly after having been accepted into the study, and another left before its completion. The latter subject and the remaining 28 study participants ranged in age between 21 and 33 years, with a mean age of 24 years. During at least the six-month period before entry into the study, all 29 subjects had smoked marihuana at least four days a week, eight had smoked marihuana daily, and four had smoked several times a day. Of the participants, 23 either had never smoked tobacco or had not smoked tobacco for six months and six smoked half a package of cigarettes or less per day. Only one subject smoked hashish regularly. Prior informed, written consent was obtained from all participants.

Subjects were sequestered on a carefully supervised research ward of the Neuropsychiatric Institute at University of California, Los Angeles, for 94 days during which multi-disciplinary studies of the biologic effects of heavy marihuana smoking, including the present investigation, were conducted. The first 11 days of confinement served as a detoxification period during which cannabis use was interdicted. Each day thereafter for the following 80 days, except for one week between the 76th and 82d days in the hospital, subjects smoked as many marihuana cigarettes as desired, but tobacco, alcohol and other drugs were not permitted. This period of daily marihuana smoking was designated as the intoxication period. Each cigarette contained 900 mg of marihuana assayed by gas-liquid chromatography at 2.2 per cent Δ9-tetrahydrocannabinol (Δ9-THC).

Serial pulmonary-function studies were performed on the eighth day of detoxification, on the fourth day ("early intoxication") and on either the 47th or the 59th day ("late intoxication") of daily ad-libitum smoking, at the end of a one-week hiatus of no smoking, which followed nine weeks of heavy daily smoking, and one month after discharge from the hospital. On each of the study days, pulmonary-function tests were performed between 9 and 10 a.m. and after at least nine hours of no smoking.

Spirometry was performed with the subject seated, with use of a 13.5-liter water-seal spirometer (Warren E. Collins, Incorporated). Forced vital capacity, forced expired volume in one second and maximal mid-expiratory flow rate were calculated from the best of at least two vital-capacity efforts. Values were considered abnormal if they were > 1.64 standard deviations below the predicted value calculated from the regression equations of Morris et al.[15] based on data obtained in nonsmoking males.

Airway resistance and thoracic gas volume were measured at or near the resting end-expiratory volume with a 900-liter, constant-volume whole-body plethysmograph.[16,17] Specific airway conductance (airway resistance divided by thoracic gas volume) was calculated from each of five sets of measurements, and the results were averaged. Values for airway resistance > 2.50 cm of water per liter per second and for specific airway conductance < 0.12 liter per second per centimeter of water per liter were considered abnormal.[16,18,19]

Closing volume was determined at least twice, a modified single-breath nitrogen technic[20] being used, with the subject standing, using a rapid-responding nitrogen analyzer (Cardio-Pulmonary Instruments, Incorporated, Model 410) and a Fleisch No. 3 pneumotachograph with electronic integration of flow. Nitrogen concentration and volume signals were recorded oscilloscopical-

From the Department of Medicine, UCLA School of Medicine, Los Angeles, CA 90024, where reprint requests should be addressed to Dr. Tashkin.

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ly, and the resulting curve was photographed. The volume at which a sustained upward deviation fom the slope of the alveolar plateau (phase III) of the nitrogen-washout curve first developed was assumed to represent closing volume, which was expressed as a per cent of vital capacity. Calculations were made from tracings in which the expired volume was within 5 per cent of the greatest vital capacity, and expiratory flow rates were maintained at approximately 0.5 liter per second. The results were then averaged. The change in nitrogen concentration per liter was calculated from the slope of the alveolar plateau.[10] Results of change in nitrogen concentration and closing volume were considered abnormal if > 2 standard deviations above the predicted value on the basis of the regression equations of Buist and Ross for nonsmoking males.[10,21]

Diffusing capacity of the lung for carbon monoxide was performed in duplicate with a single-breath technic[22]; results were considered abnormal if they were > 1.64 standard deviations below the predicted value.[23]

Results

Base-line lung function was completely normal except for abnormalities in forced expired volume in one second and maximal mid-expiratory flow rate in only one subject who smoked tobacco (half a package a day) and hashish (less than once a week), in addition to marihuana.*

Over the entire course of the study subjects smoked an average total number of 377 marihuana cigarettes and an average daily number of 5.2 cigarettes, with a range of 1.7 to 10.0 mean daily number of cigarettes per subject. In general, this degree of marihuana smoking considerably exceeded each subject's customary social usage.

The pulmonary-function tests performed after 47 or 59 days of daily smoking (but after at least nine hours of no smoking) were compared with the results of tests performed in the same subjects during the base-line detoxification period and expressed as a per cent change from the base-line values for each subject; the per cent changes for each test were then averaged for the entire group of subjects. The significance of these changes was determined with Student's t-test for paired observations; $P < 0.05$ was considered significant. The mean per cent changes in lung function ± 1 standard error (S.E.) of the mean are shown in Figure 1. Mean forced expired volume in one second fell slightly but significantly ($P<0.01$) after chronic smoking. Larger mean reductions in maximal mid-expiratory flow rate and specific airway conductance were highly significant ($P<0.001$); airway resistance increased significantly in parallel with the reduction in specific airway conductance. Individual values for maximal mid-expiratory flow rate and specific airway conductance fell in 26 and 24 subjects, respectively, out of the total group of 28 subjects. There was a slight but significant average increase in closing volume ($P < 0.05$), but nitrogen concentration per liter did not change. A modest but significant fall in diffusing capacity was also observed. Despite these findings of significant mean changes in several indexes of lung function in the direction of functional impairment, "late intoxication" results were still

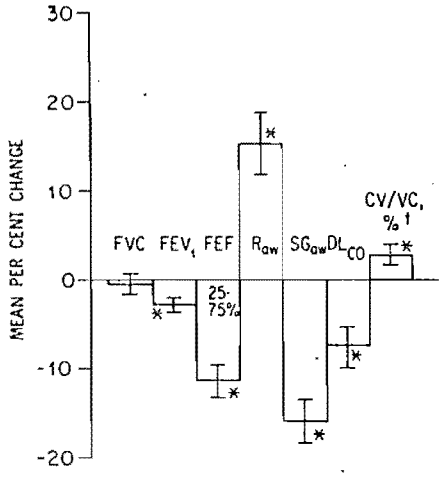

Figure 1. Changes in Forced Vital Capacity (FVC), Forced Expired Volume in One Second (FEV_1), Maximal Mid-Expiratory Flow Rate ($FEF_{25-75\%}$), Airway Resistance (R_{aw}), Specific Airway Conductance (SG_{aw}), Diffusing Capacity of the Lung for Carbon Monoxide (DL_{CO}) and Closing Volume as per Cent of Vital Capacity (CV/VC, %) after Chronic (47 to 59 Days), Heavy (Mean of 5.2 Cigarettes per Subject) Daily Marihuana Smoking in 28 Subjects.

Values and vertical bars (except for CV/VC,%) represent the means of individual per cent changes from initial base-line values ±S.E.

†Mean absolute change in CV/VC,% from base values ± 1 S.E.

*Statistically significant as compared with zero ($P<0.05$).

within the range of predicted normal values in all but three subjects, in whom maximal mid-expiratory flow rate, closing volume or airway resistance became slightly abnormal.

The average daily number of marihuana cigarettes smoked by each subject was plotted against the per cent changes in maximal mid-expiratory flow rate and specific airway conductance (Fig. 2). Linear regression slopes were obtained by the method of least squares, correlation coefficients were calculated, and the possibility of obtaining a given correlation coefficient when no correlation ex-

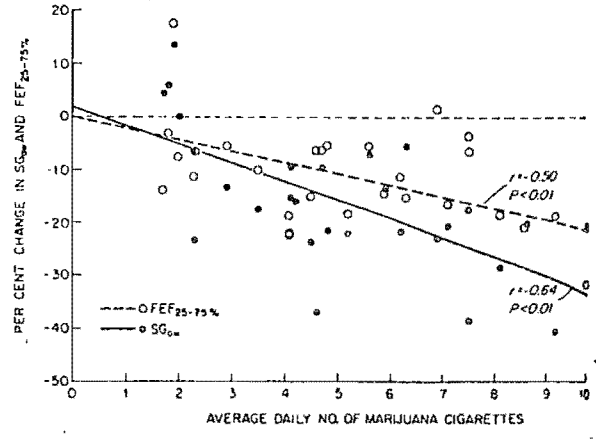

Figure 2. Relation between Average Quantity of Marihuana Smoked per Day and Reductions in Maximal Mid-Expiratory Flow Rate ($FEF_{25-75\%}$) and Specific Airway Conductance (SG_{aw}) after 47 to 59 Days in 28 Subjects.

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ined was obtained from standard probability tables. Significant correlations were found between the average quantity of daily marihuana smoked during the course of the study and the degree of reduction in mid-flow rates and specific airway conductance (Fig. 2). The changes in forced expired volume in one second and closing volume observed in each subject were not correlated with the amount of marihuana smoked.

In the subjects in whom lung-function tests were repeated both after 59 days of daily smoking and after a subsequent seven-day period of no smoking, or one month after discharge, each of the repeat studies was compared with the same subject's base-line detoxication results and expressed as a per cent change from base line. The mean per cent changes in spirometric flow rates and specific conductance for these subjects are shown in Figure 3. No significant changes in forced expired volume in one second were noted in this small subgroup of subjects. After 59 days of daily smoking, the mid-flow rates and specific conductance were significantly reduced as compared with base line. Subsequently, after one week of no smoking, the mean mid-flow rate had returned toward, but was still significantly lower than, base line, whereas specific conductance had returned essentially to base line. After one month, the mean mid-flow rate was similar to base line.

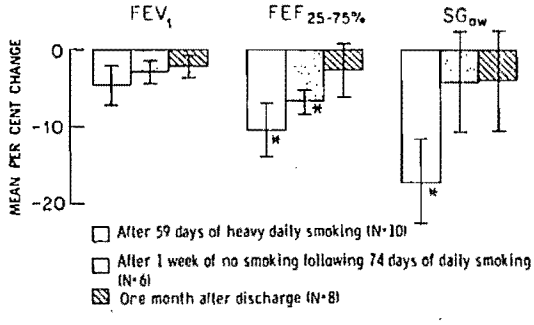

Figure 3. Changes in Forced Expired Volume in One Second (FEV_1), Maximal Mid-Expiratory Flow Rate ($FEF_{25-75\%}$) and Specific Airway Conductance (SG_{aw})with Chronic Marihuana Smoking and after Subsequent Cessation of or Reduction in Smoking.

Values and vertical bars represent the mean of individual per cent changes from initial base-line values ± 1 S.E.

*Significantly different from zero as compared with initial base-line values (paired data), $P < 0.05$.

The results of tests performed in each subject immediately after the smoking of one or more marihuana cigarettes during "early" and "late intoxication" were expressed as a per cent change from the values obtained in the same subject immediately before smoking. The average per cent changes in lung function observed in 11 subjects immediately after only one marihuana cigarette during both "early" and "late intoxication" are indicated in Figure 4. Significant differences were noted between the acute effects of one cigarette on maximal mid-expiratory flow rate, airway resistance, and specific airway conductance during "early intoxication" as compared with the same quantity smoked during "late intoxication." In 17 subjects, in whom lung function was measured immedi-

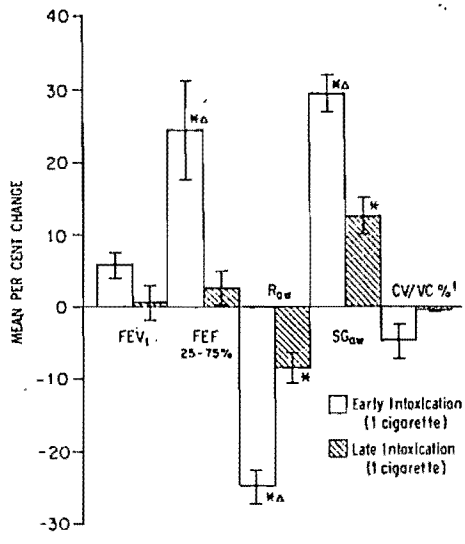

Figure 4. Acute Effects of Smoking One Marihuana Cigarette on Pulmonary Function (Abbreviations as in Figure 1) during Early (Three Days) and Late (59 Days) Marihuana Intoxication in 11 Subjects.

Values and vertical bars represent means of individual per cent changes immediately after marihuana smoking as compared with immediate pre-smoking values ± 1 S.E.

†Mean absolute change in CV/VC% from immediate pre-smoking values.

*Significantly different from zero, ($P < 0.05$).

ately after one marihuana cigarette during "early intoxication" and immediately after an average of 4.6 cigarettes during "late intoxication," comparable acute increases in forced expired volume in one second, maximal mid-expiratory flow rate, and specific airway conductance and comparable acute decreases in airway resistance were observed during both periods of intoxication.

Discussion

Previous studies have revealed definite respiratory symptoms in approximately 10 to 20 per cent of young persons who were smokers of tobacco in contrast to a virtual absence of respiratory symptoms in nonsmoking subjects of the same age.[12-14] It is of interest that none of the subjects selected for this study, 11 of whom smoked marihuana at least daily and three of whom smoked several times a day, admitted to chronic cough or sputum production, wheezing, breathlessness or frequent chest illness. However, respiratory symptoms may have been underestimated in these subjects since a standardized respiratory questionnaire was not used.

Previous studies have demonstrated significant age-adjusted differences in closing volume and maximal expiratory flow rates at middle and low lung volumes in comparisons of tobacco cigarette smokers with or without symptoms with nonsmokers.[9,11,12] Our failure to demonstrate any difference in the same indexes of lung function comparing our group of moderate-to-heavy users of marihuana with nonsmoking males[15,21] does not support the hypothesis that social degrees of marihuana smoking cause lung functional impairment. However, our study group

may not be representative of habitual marihuana smokers as a whole because it was not randomly selected.

On the other hand, the changes in lung function noted on comparison of "late intoxication" with base-line studies suggest that heavy marihuana smoking for 47 to 59 days causes mild but definite narrowing of both large and medium-sized airways (as reflected by the increases in airway resistance and decreases in specific conductance)[24] and of small airways (as reflected by the changes in maximal mid-expiratory flow rate and closing volume).[9,25] The significant correlations between the number of marihuana cigarettes smoked by any subject and the reductions in mid-expiratory flow rates and specific conductance suggest that the degree of impairment in airway dynamics is related to the quantity of inhaled irritants contained within the smoke. That these changes might be the result of an inflammatory reaction of the tracheobronchial epithelium to chronic irritation from marihuana smoke is suggested by previous findings of Henderson et al.[26] of inflammation and increased mucus on biopsies of tracheal mucosa in young men who had smoked > 25 g of hashish over several months. The improvement in lung function noted in our subjects immediately after marihuana smoking during ad libitum "late intoxication" suggests that the chronic functional changes observed are probably not due to a chronic pharmacologic effect of tetrahydrocannabinol since the tracheobronchial smooth muscle was still capable of dilating acutely in response to marihuana smoking, presumably reflecting an acute pharmacologic bronchodilator action of Δ9-tetrahydrocannabinol.[2,4] However, the significant differences in the bronchial dynamic responses to the same quantity of marihuana smoked during "late" versus "early intoxication" suggest the development of partial pharmacologic tolerance to the bronchodilator effect of tetrahydrocannabinol analogous to the tolerance found in laboratory animals to most of the pharmacologic effects of cannabis.[27]

The reduction in single-breath diffusing capacity for carbon monoxide after heavy, daily marihuana smoking was not explained by any difference in lung volume, hemoglobin concentration or time of breath-holding. Although carboxyhemoglobin levels were not measured in these subjects, it is unlikely that back pressure to the transfer of carbon monoxide was responsible for the observed decreases in the diffusing capacity of the lung for carbon monoxide[22] since carboxyhemoglobin has a half-life of approximately six hours[28] whereas our studies were performed in the morning after at least a nine-hour abstinence from smoking.

Serial lung-function tests performed in the small number of subjects who were restudied after cessation of smoking for one week and, again, one month after discharge from the hospital indicate that the mild impairment in airway dynamics resulting from several weeks of heavy daily smoking was reversible. The return of specific airway conductance to base-line values after one week of no smoking and of maximal mid-expiratory flow rate to initial values after one month of presumably less heavy smoking is consistent with a reversal of obstructive changes involving both the large and small airways after a decrease in the quantity of inhaled irritants, the improvement in smaller airways lagging behind those in larger ones. Improvement in some indexes of lung function has also been noted in tobacco cigarette smokers with mild airways obstruction one to three months after cessation of smoking.[25,29]

It is of note that despite statistically significant changes in several indexes of lung function after 47 to 59 days of heavy marihuana smoking, values were still within the predicted limits of normal in nearly all subjects, suggesting that the observed changes might not be clinically important. However, the significant correlation between the quantity of marihuana smoked and the degree of functional impairment and the lack of complete reversibility of the functional changes after one week of no smoking suggest that heavy marihuana smoking over a much longer period might lead to clinically important and less readily reversible impairment. Additional studies are needed for further definition of the nature and course of the abnormalities in lung mechanics and diffusing capacity associated with heavy marihuana smoking, to determine the relation of these physiologic abnormalities to possible morphologic changes and to evaluate the long-term effects of less heavy, social degrees of marihuana smoking on the lung.

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BIOLOGICAL DISPOSITION OF TETRAHYDROCANNABINOLS[1,2]

EDWARD B. TRUITT, JR.

Columbus Laboratories, Battelle Memorial Institute, Columbus, Ohio

Despite many centuries of use and years of research, there has been a rather large "research gap" in our knowledge about marihuana (22). A primary area of ignorance has been the almost complete lack of data concerning the fate of its cannabinoids[3] or other components in body metabolism. This situation is unfortunate because adequate drug metabolism studies are a necessary foundation for comprehensive pharmacological and toxicological tests and for rational consideration of the fundamental questions of safety and legalization.

Before the confirmation in 1964 of (−)-*trans*-Δ^9-tetrahydrocannabinol (Δ^9-THC) as the primary active component in marihuana (11), only meager evidence was available concerning the biological fate of tetrahydrocannabinols. As early as 1946, Loewe (19) showed a transfer between dogs of an ataxia-producing blood extract. Miras (23) measured the distribution of radioactivity in organs of the rat after intraperitoneal injection of purified THC preparation from a plant grown in $^{14}CO_2$. He was the first to note two striking characteristics of THC, namely that it is poorly absorbed after intraperitoneal injection, and that it is highly concentrated in the liver. With the synthesis of Δ^8- and Δ^9-THC, and the labeling of these compounds first with ^3H (5, 24), and later with ^{14}C (27), metabolic studies became possible.

Before discussing the metabolic fate of marihuana, a preliminary consideration is needed of the fate of cannabinoids during pyrolysis into marihuana smoke since this is the principal way in which the drug is used in most countries. This review will endeavor to survey the information available at present concerning the changes during the smoking process as well as after entry into the body.

SMOKING STUDIES

The earliest reported studies on the composition of marihuana smoke apparently utilized plant material having an undocumented history and did not attempt to simulate the smoking patterns typical of a marihuana smoker (7–9, 25, 30). Smoking studies at Battelle have employed a smoking machine programmed to reproduce the puff interval, rate and volume characteristics taken from a large panel of experienced smokers (Foltz *et al.*, unpublished data). Generally these smoking studies have rectified certain early misconceptions and showed that the ratios of cannabinoids in smoke are similar to the ratios of

[1] Supported by contract PH-43-68-1338 from the Center for Narcotic Addiction and Drug Abuse Studies, NIMH, and grant MH-18919, NIMH in collaboration with Roger L. Foltz, Ralph I. Mitchell, Henry M. Grotta, Allison F. Fentiman, Glenn Kinzer and E. G. Leighty.

[2] Presented in part at the First Midwestern Conference on Drug Metabolism University of Cincinnati, January, 1971.

[3] The term cannabinoid is intended to include the C_{21} compounds typical of and present

cannabinoids found in the plant. The major exception to this is that the heat of combustion converts most, but not all, of the THC-acid precursors into THC. Contrary to early speculations, there is very little conversion of cannabidiol to THC by ring closure and only minimal isomerization of Δ^9-THC to Δ^8-THC as claimed by Lerner and Zeffert (18). The recovery of Δ^9-THC which has been added to reefers, made from exhaustively extracted plant material, has not substantiated the report by Claussen and Korte (8) that over 90 % of the THC is lost through combustion. The average distribution of Δ^9-THC in smoke produced from the Battelle machine is shown in figure 1 (Foltz *et al.*, unpublished data). These data show that approximately 50 % of the total THC dose is delivered, provided the butt is fully consumed, and these findings have been confirmed in another laboratory (20).

Absorption

Many investigators have encountered difficulty in detecting, much less quantifying, unchanged THC in body fluids by using non-radioactive techniques. This difficulty is chiefly due to the rapid conversion of THC to its metabolite (*vide infra*). THC has a half-life of only about 14 min in rabbits (2), 30 min in rats (16), and roughly 30 min in man based upon experiments with three normal subjects (17).

Smoke inhalation, the principal route of administration of marihuana in man, is rapid and efficient but difficult to quantify and to apply to animals. Marihuana is also widely consumed orally, and Isbell *et al.* (15) have shown that absorption from the gastrointestinal tract is only about one-third as effective as inhalation of smoke containing THC. Many pharmacological and toxicological

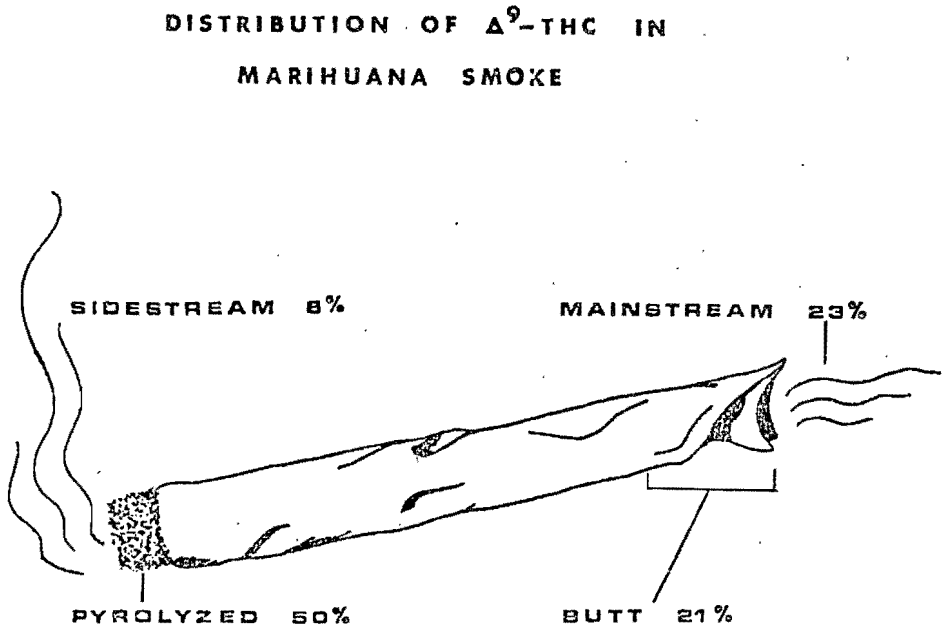


FIG. 1. Distribution of Δ^9-THC in marihuana smoke

studies point to slow and limited absorption of THC after intraperitoneal administration. The intravenous route, which is subject to limitations in chronic use and which is dissimilar to street use, remains the method of choice for short-term experiments.

The solubilization, micro-emulsification, or suspension of finely divided particles of THC is a necessary, but confounding, factor in the preparation of the drug for absorption. The agents used have included various surfactants (Tween 80, Triton X-100, Pluronic F-68), solvents (ethanol, propylene glycol, dimethylsulfoxide, glycerin, and peanut, olive, or sesame oils) and suspending agents (bovine serum albumin and gum arabic). Each of these agents may, in itself, influence the rate of absorption of THC and some may produce pharmacological effects. The wide variety of methods employed by different investigators makes quantitative comparisons difficult, and the use of oils or the suspension of unsolubilized THC microglobules appears to impede absorption.

Distribution

In addition to the early study by Miras, the distribution of radiolabeled compounds has been studied in animals (2, 12, 13, 16, 29), and in man (17). Most of these studies have used ^3H and report only the total radioactivity of tissues despite rapid metabolite formation (2, 4, 10, 32). Nevertheless, these data were useful in showing:

1. The rapid accumulation of radioactivity in the liver;
2. Biliary excretion and persistence of the drug and/or metabolites in the feces;
3. The ability of THC and/or metabolites to cross the placenta;
4. The portion of the drug and/or metabolites excreted *via* the kidney and urine;
5. The lack of preference of the drug and/or metabolites for brain tissue.

By inhalation, the distribution pattern was not remarkably different from the intraperitoneal route except for initial retention by the lung (12). In plasma, 80 to 95% of the Δ^9-THC migrates in association with lipoprotein (31).

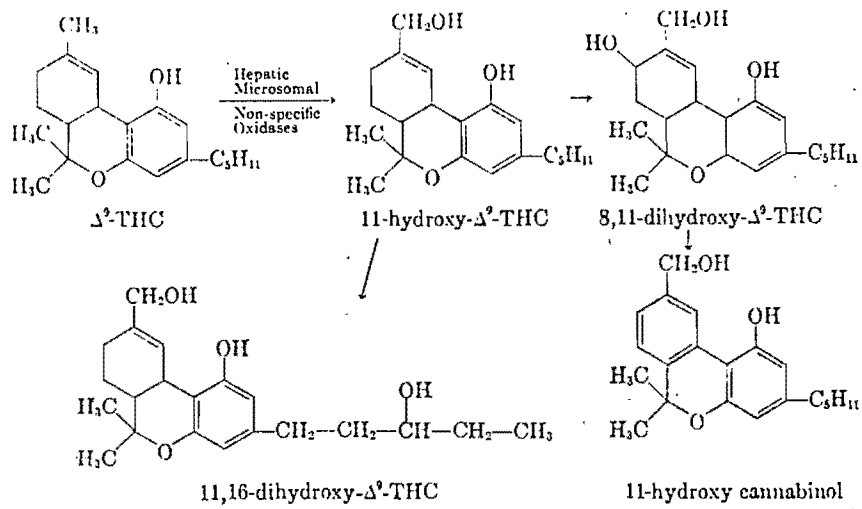
Metabolism

The hepatic accumulation noted above prompted investigators in this laboratory (10), and others (2, 4, 32), to add Δ^8- or Δ^9-THC to liver homogenates and then to the microsomal fraction to examine possible metabolism *in vitro*. Little metabolic alteration occurs in whole liver homogenates, but, when the high speed microsomal fraction (10,000 × *g*) is fortified with $NADPH_2$[4] regenerating system, a metabolite is rapidly formed which has been identified as 11-hydroxy-Δ^8-THC when Δ^8-THC was used as the precursor (4, 10), or 11-hydroxy-Δ^9-THC when Δ^9-THC was used as the precursor (24, 32). The metabolite of Δ^8-THC has also been isolated and identified in urine (4, 5).

The importance of these hydroxy-THC metabolites is emphasized by 1) their rapid formation (accounting for the difficulties of many investigators to detect

[4] $NADPH_2$, nicotinamide adenine dinucleotide phosphate-reduced form.

THC *in vivo*), 2) their pharmacological activities which strongly resemble Δ^8- and Δ^9-THC and exceed these precursors in potency, and 3) the parallel time courses of their presence and the duration of observable drug action. Wall and associates (32) have reported a further conversion of Δ^9-THC to 8,11-dihydroxy-Δ^9-THC and then to 11-hydroxy cannabinol. They have also detected sidechain oxidation at the *beta*-position. Some 11-hydroxy cannabinol is also formed from cannabinol (33). These biotransformations may be summarized in the following reactions scheme:



Undoubtedly, the biotransformation of THC is complex, and multiple products must be anticipated. However, the full significance of the metabolic events may well have other implications in addition to revealing the psychoactive metabolite. Tolerance to marihuana is suspected to decrease during initial exposures to the drug before it increases after chronic consumption of large amounts (21, 22). Thus, induction of hepatic microsomal enzymes may explain in part the increasing pharmacological effect of the drug during the first several exposures in addition to improved smoking technique and better recognition of the euphoric effect. Induction of microsomal non-specific oxidases is implied by the finding of a shortened barbiturate sleeping time in mice treated chronically with THC (28). Finally, a significant relationship may exist between persistence of dihydroxy-THC and hydroxy-cannabinol derivatives and the after-effects of THC on mood, motivation and thought processes.

Excretion

It is apparent that the apportionment of excretion products between the fecal and urinary routes will vary in different animal species (2). The presence of metabolites in the urine was detected before their identification (1, 6a). Only a very small portion of free-THC is excreted intact in the urine, but some is conjugated with glucuronic acid (2). At least three major metabolites appear in the urine of rabbits (2). These metabolites are more polar than THC and in the

case of Δ^8-THC one of these has been identified as 11-hydroxy-Δ^8-THC (4, 5). Conversion of the urinary 11-hydroxy-Δ^9-THC to cannabinol with *p*-toluenesulfonic acid may provide a method for detection of cannabis use (3).

The conversion of THC to more polar metabolites provides a method for differential extraction with diethyl ether (16). In rats, the major route of excretion for 11-hydroxy-Δ^9-THC is through the feces, whereas in rabbits urinary excretion predominates. In rats, the persistence of excretory products in the feces suggested an enterohepatic circulation which has been confirmed by Klausner and Dingell (16) who showed reabsorption of the metabolites after an intraduodenal injection of bile in an untreated recipient rat. Recycling of THC metabolites in this manner is reminiscent of the macrolide antibiotics and may also be related to the occurrence of gastrointestinal side effects by both groups of compounds.

Biological activity of metabolites

Fortuitously, a facile chemical conversion of Δ^8-THC to 11-hydroxy-Δ^8-THC has made available a supply of this metabolite in relatively pure form (>90% purity) (10). The author has examined the behavioral action of the synthetic metabolite in the rat by using a wide variety of test parameters (28) modified from Irwin's mouse behavioral screening procedures (14). The metabolite reproduces the complete pattern and time course of both Δ^8- and Δ^9-THC activities and is equal to or slightly more potent than these compounds by the intravenous route. Similar effects of all three drugs in the rat include cataleptic posturing (trance-like behavior), bizarre acts (retropulsion and backwards circling), abnormal biting, hyperstartle reactions, decreased spontaneous exploratory activity, vocalization in response to mild stimuli, and others. This same metabolite made by *de novo* synthesis also produces effects in the monkey which are similar to the parent drug (4). The metabolite of Δ^9-THC has not yet been achieved by synthesis, but relatively pure products isolated from hepatic microsomal preparations have shown THC-like behavioral activity in the mouse (24, 32). Indeed, after intracerebral injection, the potency of 11-hydroxy Δ^9-THC is about 18 times that of the precursor compound [Christensen *et al.* (6)].

On the basis of increased THC potentiation of barbital sleeping time by the microsomal metabolic inhibitor, SKF-525-A, Sofia and Barry (26) questioned the metabolite activity hypothesis at least for depressant effects of THC. However, preliminary study of this question by the author has indicated that this effect of SKF-525-A may be an influence of the drug on hepatic metabolism of barbital since the metabolite-barbital sleep time is also increased. It will be important to try the action of hydroxy-THC in man and to examine other major metabolites for possible contributions to the prolonged after effects of THC on mood, motivation and thought processes or other unsuspected toxic actions.

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Mitogen-Induced Blastogenic Responses of Lymphocytes from Marihuana Smokers

Abstract. *Blastogenic responses in vitro to phytohemagglutinin and pokeweed mitogen were examined in microcultures of peripheral blood lymphocytes from a group of 12 healthy, long-term marihuana smokers and a group of matched control subjects. With either mitogen, no significant difference in cellular incorporation of [^3H]thymidine was noted between the groups. These results were interpreted to indicate that the functional status of blood lymphocytes was not altered by long-term smoking of marihuana.*

The influence of long-term marihuana use on health is not yet clearly defined although evidence to document its potential hazards and deleterious effects on basic cellular mechanisms is accumulating. The report by Nahas and co-workers (*1*) that blastogenic responses in vitro to phytohemagglutinin and allogeneic lymphocytes were depressed in lymphocytes of long-term marihuana smokers is especially provocative. Their data indicated that the depressed responses were comparable to those of cancer and uremia patients and of transplant recipients undergoing immunosuppressive therapy. The impression gained from these data that long-term marihuana use may impair the expression of cell-mediated immunity and thus render the host more susceptible to disease is still unvalidated; the results of related studies are conflicting (*2*) and, in fact, the observations of Nahas *et al.* have not yet been confirmed directly. For these reasons, we examined the functional status of thymus-dependent (T) and -independent (B) lymphocytes from long-term marihuana smokers. However, because lymphocyte blastogenic responses have been reported to be depressed by upper respiratory infections (*3*) and by inadequate nutrition (*4*), only confirmed healthy individuals were selected for study. We report here that mitogen-induced blastogenic responses of lymphocytes from healthy, long-term marihuana smokers do not differ from those of matched control subjects.

Table 1. Mitogen-induced blastogenic responses of lymphocytes from marihuana smokers and matched control subjects; S.D., standard deviation.

Experiment	Radioactivity (dpm) per culture	
	Smokers	Controls
	Phytohemagglutinin	
1	216,418	197,306
2	163,746	167,027
3	208,781	181,150
4	155,362	163,708
5	186,119	191,547
6	128,834	125,983
7	158,440	129,687
8	202,630	202,241
9	245,436	184,572
10	221,013	141,866
11	90,166	161,611
12	168,784	147,758
Mean ± S.D.	178,811 (43,486)	166,205 (25,903)
	Pokeweed	
1	141,448	100,540
2	163,225	153,372
3	99,984	110,029
4	94,467	120,627
5	167,983	150,436
6	107,180	173,707
7	75,893	99,772
8	126,051	90,498
9	76,932	86,072
10	86,691	107,214
11	90,587	101,932
12	115,015	106,852
Mean ± S.D.	112,121 (31,535)	116,754 (27,585)

A group of 12 individuals, ranging in age from 19 to 32, who had smoked marihuana at least once per week for the previous year (average 3.4 times per week for 4.8 years) was studied. The group consisted of one black and ten white males and one white female. At the time of study, all admitted that they were still smoking marihuana and that they had smoked at least once during the preceding 48 hours. Twelve other individuals who never had smoked marihuana and denied other forms of drug abuse were selected for study as age-, sex-, and racially matched controls. All study subjects were not receiving medication at the time of study and were in good health, as judged from a detailed medical history; the values for complete blood counts, erythrocyte sedimentation, total serum protein, and serum albumin were normal. In addition, all individuals in both groups showed normal values for serum glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, alkaline phosphatase, and bilirubin and were negative on testing for hepatitis B antigen. The normal liver function tests reasonably excluded

the possibility of subclinical hepatitis—a condition known to depress cell-mediated responses (*5*)—and indirectly indicated that the subjects were not using drugs, at least those that cause abnormal liver enzyme levels (*6*).

The functional status of T and B lymphocytes was determined in vitro by their respective blastogenic responses to phytohemagglutinin-P (PHA) (Difco Laboratories) and pokeweed mitogen (PWM) (Grand Island Biological) in a microculture system similar to that described by Thurman and associates (*7*). A marihuana smoker and an appropriately matched control subject were studied within the same experiment. Lymphocyte suspensions were prepared from fresh, heparinized, peripheral blood by separation in a Hypaque-Ficoll gradient. Approximately 2×10^5 lymphocytes were cultured in 0.2 ml of RPMI 1640 medium (Grand Island Biological) containing 20 percent autologous plasma. Triplicate cultures were stimulated with 0.4 μg of either PHA or PWM; unstimulated cultures served as controls. The cultures were incubated at 37°C for 3 days when each was treated with 1 μc of [^{3}H]-thymidine (New England Nuclear) and harvested 4 hours later. Lymphocyte blastogenesis was measured by assay of cellular incorporation of radioactivity and was expressed as the average number of disintegrations per minute (dpm) per culture. The methods described by Croxton (*8*) were used for paired and unpaired *t*-tests of the significance of differences in blastogenic responses between the marihuana smokers and the matched control subjects.

The comparison of mitogen-induced blastogenic responses of lymphocytes from marihuana smokers and matched control subjects is shown in Table 1. Statistical analysis of the data by both the paired and unpaired *t*-tests confirmed that there were no significant differences ($P > .10$) in the responses to either mitogen between the groups. There also was no significant difference in [^{3}H]thymidine incorporation in the unstimulated control cultures; the respective mean values for marihuana smokers and control subjects were 735 and 737 dpm.

These results indicate that long-term marihuana smoking had no significant effect on the functional status of T and B lymphocytes and are consistent with recent evidence suggesting that chronic marihuana smokers have unimpaired immune response capabilities. They have been found to develop and exhibit delayed-type hypersensitivity responses to 2,4-dinitrochlorobenzene in the same manner as healthy nonsmokers (*9*) and even to develop humoral antibody reactivity against *Cannabis* extracts (*10*).

Our findings, however, differ completely from those of Nahas and his co-workers (*2*) who described depressed in vitro blastogenic responses in lymphocytes of marihuana smokers. Although the disagreement cannot be explained at present, it is possible that our study populations were not comparable, other than on the basis of marihuana use. They did not describe the health status of their subjects, and there was no indication in their report that they attempted to exclude subclinically ill subjects from their study, as we purposely did. Another variable to consider is the time elapsed between blood sampling and when the subjects last smoked marihuana. Because plasma levels of Δ^9-tetrahydrocannabinol, the putative active component of marihuana, reach a peak within 15 minutes after smoking and decrease rapidly thereafter (*11*), it is possible that impaired lymphocyte responses may be detectable only within a relatively short period after smoking. Even though our study subjects admitted to smoking at least once within 48 hours before study, it is likely that only a few had smoked within the 12 hours immediately preceding study. Nahas and his associates did not include this information in their report so that this possibility also remains to be evaluated. Obviously, similar immunologic studies of other populations of marihuana smokers appear necessary to clarify these divergent observations.

STEVEN C. WHITE
STEVEN C. BRIN
BERNARD W. JANICKI*
Veterans Administration Hospital, Washington, D.C. 20422

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* Address correspondence to B. W. Janicki, Allergy and Immunology Branch, Extramural Programs, NIAID, Westwood Building, Room 705, Bethesda, Md. 20016.

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DISCUSSION

The papers in this study conclusively point out two of the adverse physiological effects of marihuana use--interaction of THC with cellular membranes and pulmonary-respiratory effects. These two areas are not the only areas of concern, but there is sufficient evidence to warrant a discussion of the dangers.

The study of THC and its interaction with cellular membranes is still in its early stages. With the current medical research at the cellular level, the research on THC and membrane function will be of vital concern. Since the cell membrane is a phospholipid structure and THC is highly lipid soluble, the drug does interact with the cell membrane. Schurr (1974) found that THC inhibits glucose transport across cellular membranes of human erythrocytes.

Lysolecithin acyl transferase, a membrane-bound lymphocyte enzyme, is important in changing membrane structure. The level of this enzyme in T lymphocytes is greatly increased when foreign agents are present. Greenberg (1976) discovered that THC causes changes in the lipid phase of this enzyme, and these changes inhibit the activity of the membrane-bound enzyme.

Since smoking is the common method of marihuana use, the effect of marihuana use on the lungs is important. Tashkin (1976) found that chronic smoking of marihuana produces negative results on pulmonary function tests (forced expired volume, diffusing capacity, etc.). Leuchtenberger (1973) concluded that exposure of lung explants to marihuana smoke evokes abnormalities in DNA synthesis, mitosis, and growth. There are also changes of DNA and chromosomal complement. The exposed cultures have adverse effects such as necrosis and cell death.

Since THC persists in plasma for long periods and tends to accumulate, the toxicity of the drug is a potential danger which should be mentioned. THC is highly lipid soluble, and it has a high affinity for fat, lung, and brain tissue. At high levels the drug is toxic to cells, and this poses a potential danger for chronic users.

Based on the evidence in this study alone, there is sufficient proof that marihuana use has adverse physiological effects. The effects of marihuana use on membrane function and pulmonary function are deleterious. However, one important question remains to be answered. Does the chronic use of marihuana destroy an individual's health? To an extent, the human body is able to endure physiological stresses such as the ones discussed earlier. At this date, there is insufficient data to conclusively re-

port that chronic marihuana use is destructive to one's health. However, the ample evidence of the harmful physiological effects should serve as a warning that chronic marihuana use may be harmful to one's health.

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