ALASKA LEGISLATURE COMMITTEE FILES, 2003-2006 8672

11771 SENATE HEALTH,EDUCATION & SOCIAL SERVICES

There is a lot of evidence that the long-term effects of habitual cannabis smoking include a significantly higher prevalence of chronic and acute respiratory symptoms such as chronic cough, chronic sputum production, wheeze and acute bronchitis episodes[36 37 38 39] by comparison to non-smokers. There is evidence of a cumulative effect of smoking cannabis and tobacco in two studies[40 41] although not in another[42].

Some studies indicate that young cannabis smokers may be at risk of developing obstructive airway disease in later life[43 44]. This is supported by animal studies in which dogs[45], monkeys[46] and rats[47 48] have been exposed to varying doses of cannabis for 12-30 months and suffered damage to the smaller airway which is a major site of injury in tobacco-related COPD as well as acute and chronic pneumonia. However, other studies contradict a causal relationship between smoking cannabis and COPD[49 50 51]. Regular cannabis smoking has been associated with emphysema in some studies[52 53] but not so in others[54 55]. These studies are, however non-conclusive as they did not distinguish between smoking only cannabis and smoking cannabis together with tobacco. They also only involved a relatively small number of participants. A further study involving rats exposed to increasing doses of cannabis for 6 months did not display any evidence of emphysema although this was the case in rats exposed to tobacco smoke[56].

Further research in this area is necessary to provide more conclusive results.

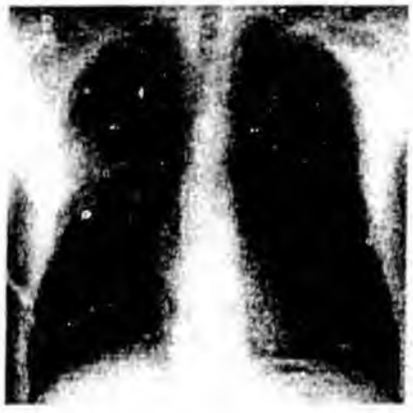
Respiratory cancer

More people die of lung cancer in the UK than from any other cancer – more than 34,000 people die every year in the UK.

As already mentioned, the tar from a cannabis cigarette contains many of the same (and even higher concentrations of) carcinogenic compounds found in cigarette smoke and deposits four times as much tar on the respiratory tract in comparison to an unfiltered cigarette of the same weight. This amplifies the exposure of cannabis smokers to particles that are known to be involved in the development of lung cancer.

There are a number of case studies (over 75) reporting cancers of the aero-digestive tract in young adults with a history of cannabis use [57 58 59 60 61 62 63] which are rare in adults under the age of 60 although the exact cause of these cancers is not clearly identifiable as many of the cases also used alcohol and tobacco. A retrospective study undertaken in the United States[64] did not find a link between smoking cannabis and tobacco-related cancers but it has been suggested that the time span investigated may not have been sufficient to study the long-term effects[65]. There is clearly a need for more epidemiological research in this area.

As it is, the development of cancer is a multistep process comprised of sequential alterations in genomic DNA (the genetic material contained in



Caption

cells) which are promoted and/ or interact with environmental and genetic factors. It is therefore often not clear what the exact cause of a particular cancer may be.

Research suggests that cannabis might contribute to cancer by manipulating the genetic makeup of cells. For lung cells to transform into cancerous cells, a specific combination of genes that regulate cell growth must be activated (in the case of oncogenes) and/ or mutate (in the case of tumour suppressor genes). THC has been shown to increase the production of a chemical particle (CYP1A1) that is responsible for causing benzpyrene (a constituent of cannabis smoke) to promote alterations in one of the most common

tumour suppressor genes, p53 thereby facilitating the development of respiratory cancer[66 67]. The gene p53 is thought to play a role in 75% of all lung cancers[68] and has been found to be expressed in 11% of individuals who smoke cannabis and tobacco[69]. Other studies looking at the effect of tar in cannabis smoke on animals[70 71 72] also indicate a correlation between cannabis and respiratory cancer.

An increase in cellular abnormalities has also been observed in cannabis smokers by comparison to non-smokers[73 74]. Abnormalities include an increase in the production of mucus producing cells (goblet cells) and reserve cells, transformation of ciliated cells into cells that resemble skin (squamous metaplasia), an accumulation of inflammatory cells and abnormalities in the cell nuclei. Nuclear alterations and squamous metaplasia have been described as precursors to the development of lung cancer in tobacco smokers[75]. In addition, smokers of cannabis and tobacco have shown a greater increase in cellular abnormalities indicating an additive effect.

Oral soft tissues

The effects of tobacco smoking on oral soft tissues have been well documented but there is little data available on the effects of cannabis smoking. However, there are some case reports that heavy cannabis use is associated with cancer of the tongue[76 77], larynx[78] and lung[79].

Other lung conditions

There have been isolated reports of spontaneous pneumothorax (breaches of the lungs causing gas in the lung cavity leading to compression of the lungs) and pneumomediastinum (breaches of the lungs causing gas in the cavities of the respiratory tract) associated temporally with the use of cannabis[80 81 82] which are thought to be caused by deep inhalation of cannabis smoke to enhance absorption of THC and hence the intoxication caused by it[83]. Deep inhalation coupled with direct pulmonary toxicity from components in cannabis in susceptible smokers has also been implicated with the formation of large lung bullae (watery blisters) in the upper respiratory area[84].

Contamination of cannabis

There has been a report of chronic cannabis smoking leading to necrotizing pulmonary granulomata (these are changes in the lungs at cellular level which may prevent the lungs from working as they should)[85] as a result of possible fungal contamination of cannabis.

Health care utilization by cannabis smokers

This has been assessed in an epidemiological study in which cannabis smokers who had never smoked tobacco were compared with non-smokers[86]. Frequent cannabis smokers showed a slight increase in outpatient visits for respiratory and other illnesses compared with non-smokers as well as a small increased risk of hospitalization.

Potential therapeutic benefits

The bronchodilator effects of THC in cannabis have also been found in the case of asthmatics with mild to moderate airway obstruction although not to the same extent as in healthy people[87]. This has led to suggestions that THC might have therapeutic benefits in asthma. However, the noxious gases, chronic airway irritation or malignancy after long-term use associated with smoking would seem likely to negate these benefits over the long term. Oral intake of THC has also shown to cause unwanted side-effects such as central nervous system intoxication and an excessive increase in heart rate[88 89]. Furthermore, tolerance to the bronchodilator effects of THC has been demonstrated after several weeks of use[90].

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Pulmonary hazards of smoking marijuana as compared with tobacco.

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To compare the pulmonary hazards of smoking marijuana and tobacco, we quantified the relative burden to the lung of insoluble particulates (tar) and carbon monoxide from the smoke of similar quantities of marijuana and tobacco. The 15 subjects, all men, had smoked both marijuana and tobacco habitually for at least five years. We measured each subject's blood carboxyhemoglobin level before and after smoking and the amount of tar inhaled and deposited in the respiratory tract from the smoke of single filter-tipped tobacco cigarettes (900 to 1200 mg) and marijuana cigarettes (741 to 985 mg) containing 0.004 percent or 1.24 percent delta 9-tetrahydrocanabino As compared with smoking tobacco, smoking marijuana was associated with nearly fivefold greater increment in the blood carboxyhemoglobin level, an approximately threefold increase in the amount of tar inhaled, and retention i the respiratory tract of one third more inhaled tar (P less than 0.001). Significant differences were also noted in the dynamics of smoking marijuan and tobacco, among them an approximately two-thirds larger puff volume, a one-third greater depth of inhalation, and a fourfold longer breath-holding tir with marijuana than with tobacco (P less than 0.01). Smoking dynamics and the delivery of tar during marijuana smoking were only slightly influenced b the percentage of tetrahydrocanabinol. We conclude that smoking marijuana, regardless of tetrahydrocannabinol content, results in a substantially greater respiratory burden of carbon monoxide and tar than smoking a similar quanti of tobacco.

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Pulmonary complications of smoked substance abuse.

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After tobacco, marijuana is the most widely smoked substance in our society Studies conducted within the past 15 years in animals, isolated tissues, and humans indicate that marijuana smoke can injure the lungs. Habitual smokin of marijuana has been shown to be associated with chronic respiratory tract symptoms, an increased frequency of acute bronchitic episodes, extensive tracheobronchial epithelial disease, and abnormalities in the structure and function of alveolar macrophages, key cells in the lungs' immune defense system. In addition, the available evidence strongly suggests that regularly smoking marijuana may predispose to the development of cancer of the respiratory tract. "Crack" smoking has become increasingly prevalent in our society, especially among habitual smokers of marijuana. New evidence is emerging implicating smoked cocaine as a cause of acute respiratory tract symptoms, lung dysfunction, and, in some cases, serious, life-threatening act lung injury. A strong physician message to users of marijuana, cocaine, or bc concerning the harmful effects of these smoked substances on the lungs and other organs may persuade some of them, especially those with drug-related respiratory complications, to quit smoking.

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Concurrent tobacco and marijuana use may hamper cigarette smoking cessation

Tobacco smokers who also smoke marijuana may be less likely to quit smoking tobacco and less likely to try to quit than those who do not smoke marijuana, according to a study by researchers at The Johns Hopkins University. Dr. Daniel Ford and colleagues interviewed 431 adults who had reported being current tobacco smokers in a study conducted 13 years earlier. In the baseline interview, more than 40 percent of the participants reported having smoked marijuana, with more than 25 percent reporting using it within the previous 30 days (recent use) and nine percent reported daily use for two weeks or more. At the 13-year follow-up, 79 percent of participants who had reported smoking tobacco at baseline were still smoking it.

Recent and daily use of marijuana at baseline were more predictive of continued tobacco smoking than use of marijuana more than a month prior to baseline. Participants who reported recent use were about twice as likely to continue to smoke tobacco 13 years later compared those who did not use marijuana within the preceding 30 days. Those who reported daily marijuana use were over three times more likely to still smoke tobacco. About 66 percent of recent marijuana users reported trying to quit tobacco during the following 13 years compared to 80 percent of those who had never used marijuana.

WHAT IT MEANS: These findings suggest that marijuana use may interfere with tobacco cessation attempts. However, there is no evidence that marijuana use can substitute for tobacco use.

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This study, funded by the National Institute on Drug Abuse, was published in the August 2002 issue of Drug and Alcohol Dependence.

The National Institute on Drug Abuse (NIDA) is a component of the National Institutes of Health, U.S. Department of Health and Human Services. NIDA supports more than 85 percent of the world's research on the health aspects of drug abuse and addiction. The Institute carries out a large variety of programs to ensure the rapid dissemination of research information and its implementation in policy and practice. Fact sheets on the health effects of drugs of abuse and other topics are available in English and Spanish. These fact sheets and further information on NIDA research and other activities can be found on the NIDA home page at http://www.drugabuse.gov.

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Science Blog

April 2003

From NIH/National Institute on Drug Abuse

Concurrent tobacco and marijuana use may hamper cigarette smoking cessation

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The pharmacological activity of inhalation exposure to marijuana smoke in mice

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Abstract

Although the majority of cannabinoid users smoke marijuana, the preponderance of laboratory animal research is based on administration of Δ^9-tetrahydrocannabinol (Δ^9-THC) or other cannabinoid agents via injection. The aim of the present study was to evaluate the impact of inhaling marijuana, or ethanol-extracted placebo smoke in the mouse model of cannabinoid activity by assessing inhibition of spontaneous activity, antinociception, catalepsy, and body temperature. In order to determine dosimetry, blood levels of Δ^9-THC were obtained following either marijuana exposure or intravenous injection of Δ^9-THC. Inhalation exposure to marijuana produced dose-related increases in antinociception and catalepsy, with estimated ED_{50} doses of Δ^9-THC of 2.4 and 3.8 mg/kg, respectively. However, hypothermia and locomotor depression occurred in both the placebo- and marijuana-exposed mice. The CB_1 receptor antagonist, SR 141716A antagonized the antinociceptive effects of marijuana (AD_{50}=0.6 mg/kg), but only slightly decreased marijuana-induced catalepsy, and failed to alter either the hypothermic or locomotor depressive effects. In contrast, SR 141716A antagonized the antinociceptive, cataleptic, and hypothermic effects of intravenously administered Δ^9-THC in mice that were exposed to air alone, though all subjects exhibited locomotor depression, possibly related to the restraint. In accordance with reports of others, these data suggest that exposure to smoke alone has

pharmacological consequences. Our findings also indicate that marijuana-induced antinociception is mediated through a CB_1-receptor mechanism of action and are consistent with the notion that Δ^9-THC is mainly responsible for this effect.

Author Keywords: Marijuana smoking; Cannabinoid; Δ^9-THC; SR 141716A; Antinociception; Analgesia; Catalepsy; Hypothermia

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Researchers At UCLA's Jonsson Cancer Center Report Smoking Marijuana May Increase Risk Of Head And Neck Cancers

Researchers at UCLA's Jonsson Cancer Center are reporting, for the first time, that smoking marijuana may increase the risk of head and neck cancers.

Results of a . epidemiological study of more than 340 people are outlined in an article published in today's (Dec. 17) edition of the peer-reviewed journal Cancer Epidemiology Biomarker and Prevention.

Previous laboratory and clinical studies have indicated that marijuana use may be related to molecular alterations in the respiratory tract, changes that may lead to cancer. This is the first study to examine whether smoking marijuana increases risk of head and neck cancers, said Dr. Zuo-Feng Zhang of UCLA's Jonsson Cancer Center, a professor in the Department of Epidemiology in the UCLA School of Public Health and director of the cancer epidemiology training program at UCLA.

"Most people don't think about marijuana in relationship to cancer," said Zhang, lead author of the journal ar' le. "The carcinogens in marijuana are much stronger than those in tobacco. The big message here is that marijuana, like tobacco, can cause cancer."

Zhang studied the relationship between marijuana use and head and neck cancers in 173 patients diagnosed with those diseases. He compared those findings to 176 cancer-free control patients, and found that those who habitually smoked marijuana were at higher risk for head and neck cancers.

The epidemiological data was collected using a structured questionnaire, which queried patients about their histories of tobacco smoking, marijuana smoking and alcohol use. Zhang said researchers were able to evaluate the data on marijuana smoking independently from data on tobacco smoking and alcohol use, which also increase the risk of certain cancers.

The results of the study are particularly important now, Zhang said, as habitual marijuana smokers from the 1960s reach older ages. Because head and neck cancers -- cancers of the mouth, tongue, larynx and pharynx -- take many years to develop, people who smoked large amounts of marijuana in the 1960s may just now be contracting head and neck cancers, Zhang said.

"In the '60s, we had very high numbers of people in their 20s smoking marijuana," Zhang said. "These people are just now getting to the ages at which they will get head and neck cancers. This is the time to study a risk like this."

The more times per day a person smokes marijuana, the greater his or her risk of head and neck cancers, according to the study. Additionally, people who use marijuana habitually for many years also increase their risk of head and neck cancers, Zhang said.

Congressional - M. Moustakas Cerebrovascular disease

"If you smoke a little, your risk increases a little," Zhang said. "If you smoke a lot, your risk increases a lot."

Marijuana is the most commonly used illegal drug in the United States, Zhang said. It is estimated that about 31 percent of the U.S. population 12 years or older has used marijuana, according to the journal article.

Zhang's research builds on previous studies of marijuana and cancer risk. An article by UCLA cancer researchers published in the Aug. 19, 1998, issue of the Journal of the National Cancer Institute stated that habitual smoking of marijuana and crack cocaine causes the same kinds of molecular changes that precede the development of lung cancer in cigarette smokers.

"Now we have evidence that may link marijuana smoking to head and neck cancers," Zhang said. "Many people may think marijuana is harmless, but it's not."

In addition, the epidemiological study and the subsequent journal article also touch on the interplay between marijuana smoking and the genetic defect that prevents DNA from repairing itself. Some marijuana smokers with this genetic defect might not have the ability to repair DNA damage prompted by the habit. Zhang said these people are about 16 times more likely to develop head and neck cancers than non-marijuana smokers whose DNA repair function is operating normally.

Zhang said larger epidemiological studies are needed to replicate the results obtained by UCLA cancer researchers. One such study, funded by the National Institutes of Health, is being conducted now at UCLA.

This story has been adapted from a news release issued by University Of California, Los Angeles Health Sciences.

Cancer Epidemiology Biomarkers & Prevention Vol. 8, 1071-1078, December 1999

Marijuana Use and Increased Risk of Squamous Cell Carcinoma of the Head and Neck[1]

Zuo-Feng Zhang[2], Hal Morgenstern, Margaret R. Spitz, Donald P. Tashkin, Guo-Pei Yu, James R. Marshall, T. C. Hsu and Stimson P. Schantz

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Abstract

Marijuana is the most commonly used illegal drug in the United States. In some subcultures, it is widely perceived to be harmless. Although the carcinogenic properties of marijuana smoke are similar to those of tobacco, no epidemiological studies of the relationship between marijuana use and head and neck cancer have been published. The relationship between marijuana use and head and neck cancer was investigated by a case-control study of 173 previously untreated cases with pathologically confirmed diagnoses of squamous cell carcinoma of the head and neck and 176 cancer-free controls at Memorial Sloan-Kettering Cancer Center between 1992 and 1994. Epidemiological data were collected by using a structured questionnaire, which included history of tobacco smoking, alcohol use, and marijuana use. The associations between marijuana use and head and neck cancer were analyzed by Mantel-Haenszel methods and logistic regression models. Controlling for age, sex, race, education, alcohol consumption, pack-years of cigarette smoking, and passive smoking, the risk of squamous cell carcinoma of the head and neck was increased with marijuana use [odds ratio (OR) comparing ever with never users, 2.6; 95% confidence interval (CI), 1.1–6.6]. Dose-response

relationships were observed for frequency of marijuana use/day (P for trend < 0.05) and years of marijuana use (P for trend < 0.05). These associations were stronger for subjects who were 55 years of age and younger (OR, 3.1; 95% CI, 1.0–9.7). Possible interaction effects of marijuana use were observed with cigarette smoking, mutagen sensitivity, and to a lesser extent, alcohol use. Our results suggest that marijuana use may increase the risk of head and neck cancer with a strong dose-response pattern. Our analysis indicated that marijuana use may interact with mutagen sensitivity and other risk factors to increase the risk of head and neck cancer. The results need to be interpreted with some caution in drawing causal inferences because of certain methodological limitations, especially with regard to interactions.

Introduction

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Marijuana is the second most commonly smoked substance in the United States after tobacco (1 , 2) . It is estimated that 31% of the United States population 12 years or older in 1992 had ever used marijuana (3) . Studies conducted within the past two decades in experimental animals and humans indicate that marijuana smoke can injure the lung and respiratory tract (4) . In humans, habitual smoking of marijuana has been shown to be associated with symptoms of chronic bronchitis, an increased frequency of acute bronchitic episodes, extensive tracheobronchial epithelial histopathology including alterations correlated with the subsequent development of malignancy in tobacco smokers (5) , DNA injury (6) , and abnormalities in the structure and function of alveolar macrophages, key cells in the immune defense system of the lung (7 , 8) . Further evidence also suggests that marijuana may predispose to the development of cancer of the respiratory tract (9) . For example, the tar phase of marijuana smoke contains some of the same carcinogenic compounds found in tobacco smoke, such as phenols and polycyclic aromatic hydrocarbons, including benzo[*a*]pyrene, which is present in ≈50% higher concentration in marijuana tar than in the tar from a comparable amount of unfiltered tobacco (10) . In addition, a single marijuana cigarette deposits four times as much tar in the respiratory tract as that deposited from a single filtered tobacco cigarette of approximately the same weight (11) . The higher content of carcinogenic polycyclic aromatic hydrocarbons in marijuana smoke and the greater deposition of marijuana tar in the lung act together to amplify exposure of the marijuana smoker to carcinogens in the particulate phase. Finally, preliminary *in vitro* studies involving mixed reactions of antigen-presenting dendritic cells and T lymphocytes (12) and *in vivo* studies using a murine model of an immunogenic carcinoma of the lung (12 , 13) suggest that Δ9-tetrahydrocannabinol, the major psychoactive ingredient in marijuana smoke, impairs immune responses to tumor antigens. A recent paper reported that habitual marijuana (and/or cocaine) smokers exhibited more molecular genetic abnormalities than nonsmokers (14) . The study suggested that smoking marijuana and or cocaine, like tobacco smoking, exerts field cancerization effects on bronchial epithelium, which may place marijuana/cocaine smokers at increased risk for the subsequent development of lung cancer.

The above-cited biological evidence pointing to a carcinogenic role for marijuana is supported by several case-series reports, indicating an unexpectedly large proportion of marijuana users among

selected cases of upper aerodigestive tract cancer. Since 1986, a total of 74 anecdotal cases of head and neck cancer with a history of marijuana use have been reported in medical literature (15, 16, 17, 18, 19, 20, 21, 22, 23, 24) . The characteristics of these marijuana-exposed malignancies of the upper aerodigestive tract include young age at diagnosis (<55 years old), extensive field cancerization, and aggressive biological behavior. Although causal inference cannot be made directly from uncontrolled case-series studies, these case reports suggest a need for in-depth epidemiological investigations of the relationship between marijuana use and the risk of cancers of the upper aerodigestive tract.

In the only published epidemiological study of marijuana use and cancer incidence, the authors reported positive associations between lifetime marijuana use (six or more occasions) and both prostate and perhaps cervical cancer among nonsmokers of tobacco cigarettes. No association was observed between marijuana use and all tobacco-related cancers (25) . Unfortunately, the specific relationship between marijuana use and cancers of the head and neck, those sites most likely to be affected by marijuana use along with lung, was not explored independently. Moreover, subjects in the latter study (25) may not have been followed long enough for adequate assessment of an effect of marijuana on cancer risk. In addition, there may not have been enough exposure to marijuana to observe an effect in this population.

The aim of the present case-control study was to examine the association between marijuana use as derived from questionnaire data and head and neck cancers, controlling for other known risk factors for the disease, including cigarette smoking and alcohol drinking. We also examined the possible gene-environment interaction between marijuana use and mutagen sensitivity, as well as interactions with other known risk factors for head and neck cancer. Mutagen sensitivity is considered a predisposition marker of cancer risk (26, 27, 28, 29) . Defects in one or more steps of the DNA repair process may play a significant role in environmental carcinogenesis, and the extent of such defects may be partially responsible for susceptibility or resistance to environmental mutagens (30) . Mutagen sensitivity tests are indirect indicators of DNA repair competence. Bleomycin, a radiomimetic agent, was used as the test mutagen to evaluate the rates of induced chromosome breakage as a crude indicator of the response to a genotoxic agent (31 , 32) .

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Cases and Controls.

Untreated new patients with a histologically confirmed diagnosis of first primary squamous cell carcinoma of the head and neck, seen at Memorial Sloan-Kettering Cancer Center from 1992 to 1994, were considered as cases in this study. We approached 192 patients, and 173 agreed to participate. Sites of disease were classified by the American Joint Committee on Cancer criteria and coded by the International Classification of Diseases Version 9 (ICD-9). The tumor sites included lip (ICD-9, 140; n = 2), tongue (ICD-9, 141; n = 52), salivary glands (ICD-9, 142; n = 1, metastatic lesion, squamous cell carcinoma), gum (ICD-9, 143; n = 13), floor of mouth (ICD-9, 144; n = 15), other parts of the mouth (ICD-9, 145; n = 11), oropharynx (ICD-9, 146; n = 12), nasopharynx (ICD-9, 147; n = 2, squamous cell carcinomas), hypopharynx (ICD-9, 148. n = 13), other oral cavity (ICD-9, 149; n = 2), esophagus (ICD-

9, 150; $n = 1$), nasal cavities (ICD-9, 160; $n = 1$), and larynx (ICD-9, 161; $n = 48$). Age- and sex-frequency matched controls were identified for this study. Controls were without a history of cancer and were identified from the Blood Bank Center of Memorial Sloan-Kettering Cancer Center during the same period. We approached 196 blood donors, and 176 agreed to participate in the study.

Data Collection.

The study was approved by the Institutional Research Board on Human Subjects of Memorial Sloan-Kettering Cancer Center. All cases and controls were asked to sign an informed consent form if they agreed to participate in the study, to complete a structured questionnaire, and to donate a sample of blood. The questionnaire requested information on the following variables: age, gender, race, year and place of birth, religion, family income, and education; average number of tobacco cigarettes smoked/day, years of smoking, age at initiation of smoking; exposure to environmental tobacco smoking (at home and at work); alcohol consumption, types and frequency of alcohol consumption; occupational and environmental exposures; family history of cancer; sexual history; medical history; and oral hygienic history. In addition, all subjects were asked if they had ever used marijuana. If they responded yes, subjects were asked the average number of times they smoked/day and the number of years of marijuana use.

Mutagen-Sensitivity Assay.

A total of 91 patients and 131 controls provided a blood specimen for the assessment of mutagen sensitivity. The mutagen-sensitivity assay used in this study has been described in detail previously (33) . A peripheral blood sample (10 ml or less) was collected from cases and controls in a heparinized tube prior to initiation of lymphocyte culture. The standard lymphocyte culture procedure used RPMI 1640, supplemented with 15% FCS and phytohemagglutinin, in a ratio of blood:medium of 1:9. At 67 h of incubation, one set of cultures was treated with bleomycin (0.03 unit/ml) for 5 h. Colcemid (0.04 mg/ml) was added in the last hour to induce mitotic arrest prior to harvesting. A conventional cell-harvesting procedure followed. The cells were treated with hypotonic KCl (0.975 M KCl) solution for 15–20 min, fixed, washed with a freshly prepared mixture of methanol:acetic acid (3:1), and air-dried on wet slides. The slides were stained with Giemsa solution without banding. Fifty well-spread metaphases were examined from coded slides. Chromatid aberrations recorded were frank chromatid breaks or exchanges. Bleomycin tends to induce few chromatid exchanges (which, if present, are considered as two breaks). Chromatid gaps or attenuated regions were disregarded. The frequency of breakage was expressed as breaks/cell. The reliability of cytogenetic scoring has been evaluated previously by comparing four separate blood samples from a respective donor with a minimum interval between samples of 1 week. Mutagen sensitivity appeared to be stable and representative in a random-effect, one-way ANOVA model (30) .

Statistical Analysis.

The effects of marijuana use on the risk of head and neck cancer were estimated with ORs[3] and their 95% CIs, derived from logistic regression analysis (34) . Continuous variables, such as years of marijuana use and frequency of use, were first analyzed as continuous variables and then divided into three groups according to their marginal distributions: frequency of use (marijuana use/day) was categorized as never, less or equal to once per day, and more than once per day; and years of use was

categorized as never use, 1–5 years, and >5 years. For eight cases and nine controls who reported previous marijuana use but failed to report frequency of use, the median value of once per day was used to replace the missing values for the continuous variable and for the categorical variable. For five cases and five controls who reported previous use but provided no information on years of use, the median value of 5 years was used for the continuous variable and 1–5 years category for the categorical variable. Results of both replacing missing data with median values and excluding missing data are presented in the results. Dummy variables were used in logistic regression analysis to estimate ORs for each category of exposure. Trend tests for ordered variables were performed by assigning the score j to the jth exposure level of a categorical variable (where $j = 1, 2, \ldots$) and treating the categorical variable as an interval predictor in unconditional logistic regression. Three models were used to assess marijuana effects: (*a*) no covariates (crude analysis); (*b*) statistical adjustment for pack-years of cigarette smoking (continuous variable); (*c*) statistical adjustment for pack-years of cigarette smoking plus age (continuous variable), sex (male, female), race (white, nonwhite), education (≤high school, college, >college), passive smoking (no, yes), and heavy alcohol drinking (<100 drinks/month; ≥100 drinks/month). Stratified analysis was used to assess departures from additive effects between marijuana use and other known risk factors for head and neck cancer, including cigarette smoking, alcohol drinking, and mutagen sensitivity.

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The overall prevalence of lifetime marijuana use was 9.7% in controls and 13.9% in cases. The highest prevalence of marijuana use was found in cases with squamous cell carcinoma of the larynx ($n = 48$; 22.9%) and tongue ($n = 52$; 19.2%). The distributions of marijuana use among cases and controls, stratified by demographic characteristics, cigarette smoking, alcohol drinking, and mutagen sensitivity, are shown in Table 1⊡. Age was strongly associated with marijuana use; large proportions of marijuana smokers were found in younger age groups for both cases and controls. No obvious differences in marijuana use were found between categories of gender, race, or education. Tobacco cigarette smoking was generally independent of marijuana use in both cases and controls, except for those variables related to age such as pack-years of tobacco cigarette smoking, years of smoking, and age at smoking initiation in cases. Heavy alcohol drinking and mutagen hypersensitivity were not related to marijuana use in cases or controls. Passive smoking, not associated with marijuana use in controls, was related to marijuana use in cases.

View this table: [in this window] [in a new window] Table 1 The distribution of marijuana use (number and percentage of users and nonusers) in cases and controls, by category of selected demographic factors, smoking, alcohol, and mutagen sensitivity

The estimated crude OR for the effect of lifetime marijuana use (ever *versus* never) on the risk of head and neck cancer was 1.5 (95% CI, 0.8–2.9). Adjusting for age, gender, race, education, heavy alcohol

drinking, pack-years of tobacco cigarette smoking, and passive smoking increased the OR to 2.6 (95% CI, 1.1–6.6; Table 2). Strong dose-response relationships were observed for the effects of frequency of marijuana use and years of use. The adjusted ORs were 2.1 for those who smoked marijuana once per day and 4.9 for those who smoked marijuana more than once per day (P for trend = 0.0358) when missing values were replaced by median values. After excluding those with missing information on frequency of marijuana use, the adjusted ORs were 4.0 (0.9–2.4) and 5.4 (0.9–33) for those who smoked once per day and more than once per day, respectively (P for trend = 0.0214). Of those who smoked marijuana for 1–5 years, the adjusted OR was 1.9 (0.6–5.9); for individuals who smoked marijuana >5 years, the adjusted OR was 4.3 (0.99–19) when missing values were replaced by median values (P for trend = 0.0325; Table 2). After excluding those with missing information on years of marijuana use, the adjusted ORs were 3.9 (0.99–15) and 4.9 (0.8–29) for those who smoked 1–5 years and >5 years, respectively (P for trend = 0.0134).

View this table: [in this window] [in a new window] Table 2 Estimated effects of marijuana use (OR and 95% CI) on the risk of head and neck cancer, by covariates selected for adjustment[a]

The observed association between marijuana use and head and neck cancer was stronger among younger subjects. When the analyses were restricted to 55 cases and 65 controls <55 years, the adjusted OR increased to 3.1 (95% CI, 1.0–9.7; Table 3). Dose-response relationships were also stronger for the effects of frequency of marijuana use and years of use, controlling for the same covariates. When the analysis was further restricted to those between the ages of 40 and 55, the magnitudes of the estimated effects were still persistent. No association was observed between marijuana use and head and neck cancer for those 55 years or older.

View this table: [in this window] [in a new window] Table 3 Estimated effects of marijuana use (OR and 95% CI) on the risk of head and neck cancer for individuals <55 years by covariates selected for adjustment[a]

Table 4 shows the combined effects of lifetime marijuana use (ever *versus* never) and each of three potential effect modifiers: tobacco cigarette smoking, alcohol use, and mutagen sensitivity. For these analyses, we used >1.0 breaks/cell as the cutoff value to define hypersensitivity and having 100 or more drinks per month as a cutoff point for heavy drinking, and we categorized tobacco cigarette smoking into never smoking, former smoking, and current smoking. These variables were further stratified by marijuana use. The effects of marijuana use and cigarette smoking were more than multiplicative; the adjusted OR for the joint category of current tobacco cigarette smokers and marijuana users was greater than the product of the two component effects for those two exposures, *i.e.*, $36.1 > 3.6 \times 2.6 = 9.4$. Similar interaction effects (greater than multiplicative) were found for marijuana use and mutagen sensitivity. The adjusted OR for joint category of marijuana use and mutagen sensitivity was greater than the product of two component effects, *i.e.*, $77.1 > 6.1 \times 1.1 = 6.7$. The effects of marijuana use and

alcohol consumption appeared more than additive but less than multiplicative, *i.e.*, 4.3 (alcohol only) + 2.7 (marijuana only) - 1 = 6.0 < 9.6 (both exposures) < 4.3 x 2.7 = 11.6. In each case, however, power for testing each null hypothesis (effects are additive or multiplicative) and for comparing the fits of additive *versus* multiplicative models was low.

View this table: [in this window] [in a new window] Table 4 Estimated combined effects (OR and 95% CI) of lifetime marijuana use (ever *versus* never) and each of three potential modifiers (cigarette smoking, heavy alcohol use, and mutagen hypersensitivity) on the risk of head and neck cancers by covariates selected for adjustment

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This study has several possible limitations. One limitation is potential selection bias, which might have resulted in an overestimate of the marijuana effect (bias away from null). The controls for this study were blood donors and possibly less likely to be substance abusers. If use of marijuana were inversely associated with blood donation, the selection bias would lead to an overestimate of the marijuana effect. The blood donors at Memorial Sloan-Kettering Cancer Center had to be between the ages of 17 and 75, weigh 110 pounds or more, and be in good health. The prospective donors were asked to give a health history and take a physical examination to ensure the greatest possible safety for both donors and recipients. Nevertheless, the only question directly related to drug abuse was: "Have you used illegal drugs with a needle?" Although marijuana is not generally injected, marijuana use and injected drug use could be positively associated, which might lead to an overestimate of the marijuana effect. Nevertheless, because the observed prevalence in our controls was similar to the expected prevalence based on national data, the selection of blood donors as controls probably did not affect the association under study (Table 5).

View this table: [in this window] [in a new window] Table 5 Lifetime prevalence (%) of marijuana use in the study controls (no. of user /total) and the United States population, 12 years and older, from 1991 to 1993, by birth cohort[a]

When we evaluated the interaction between marijuana smoking and mutagen sensitivity (Table 4), the possible selection bias might exist because those with blood samples for mutagen assay may be different from individuals without blood samples. A total of 26.1% of controls and 46.8% of cases refused to provide a blood sample for the bleomycin in this study. We have compared the differences between those with and without blood samples on selected variables. This attempt is crucial to show whether there is selection bias attributable to missing samples that may threaten the validity of the interaction between marijuana smoking and mutagen sensitivity. No obvious difference was found between those with and without blood samples in terms of age, gender, race, education, and marijuana smoking. Only a board-line difference for cigarette smoking between those with and without blood samples in head and

neck cases was detected ($P = 0.05$), which indicates that the subjects with blood samples might not be a selected group for smoking habits from the original study population. The association between tobacco smoking and risk of head and neck squamous cell cancer was stronger in people with mutagen data then those without mutagen data, which might lead to a stronger confounder effects on the association between marijuana smoking and head and neck cancer. However, when the interaction between mutagen sensitivity and marijuana use was evaluated, the point estimates of the crude ORs were pretty similar to those after controlling for pack-years (Table 4).

A second limitation is differential misclassification of marijuana use, which may also bias the estimated marijuana effect. Because marijuana smoking is illegal, cases and controls might tend to underreport their history of marijuana use, but the degree of underreporting might have been greater for controls than cases who might want to rationalize their disease. Thus, the estimates of marijuana effects could be positively biased. On the other hand, cancer patients, under some duress because of their illness, could underreport their history of marijuana use more than controls, which would negatively bias the estimated marijuana effects. To address this potential source of bias, we compared the reported lifetime prevalence of marijuana use in controls with the corresponding prevalence in the United States population during the same period, stratified by gender and year of birth (Table 5; Ref. 3). We found that the overall (crude) lifetime prevalence of marijuana use in each gender of the controls was approximately equal to the corresponding prevalence in the United States population standardized to the birth-cohort distribution of the controls. For the majority of controls born before 1951 ($n = 152$; 86%), the lifetime prevalence of marijuana use was similar to estimates for the United States population. For a small fraction of those controls born since 1951 ($n = 24$; 14%), however, there is some indirect evidence for systematically underreporting of marijuana use. When we reanalyzed the data by excluding those cases and controls born since 1951, we found little change in the estimated marijuana effects. Because we cannot address issues of either over- or underreporting by cases, it is difficult to evaluate the direction of bias by differential misclassification of past marijuana use on the association under study. The possible limitation of using mutagen sensitivity assays in case-control study was discussed by Caporaso (29) . Cultured cells obtained from patients with cancer or control subjects in a hospital setting can differ for abnormal nutrition, secondary metabolic alterations of neoplastic disease, and effect of treatment, hospitalization, inactivity, or stress, which will allow bias attributable to differential misclassification. However, a recent paper by Cloos *et al.* (28) reported a high heritability estimate of the susceptibility to bleomycin-induced chromatid breaks, which indicates that a clear genetic basis for mutagen sensitivity-related cancer susceptibility may exist in the general population. If the mutagen sensitivity is highly inherited, the differential misclassification bias for this assay might be minimal.

The third limitation is low power and precision. The relatively small sample size and low frequency of marijuana use limits our ability to estimate the effects precisely, especially when analyzing specific sites or when assessing interaction effects with other risk factors.

A fourth possible source of bias is no differential error in measuring confounders of the association under study. It has been shown, for example, that no differential misclassification of a strong confounder will cause the investigator to underestimate both the impact of the confounder on effect estimate and the

association of the confounder with the factor under study (35 , 36) . However, even if the association of major confounders, such as alcohol and tobacco with marijuana, are stronger than they appear, they appear so weak as to represent an unlikely source of bias.

Possible confounding effects also need to be addressed. We have evaluated the possible confounding effects to identify the potential confounders that induced the large changes in point estimates of ORs and *P*s. Our results showed that age was a major confounder, which causes the largest changes in point estimates of OR and *P* for marijuana smoking after controlling for it. In addition, passive smoking and pack-years of smoking are positive confounders, and alcohol drinking is a negative confounder on the association between marijuana and head and neck cancer.

This is the first epidemiological study to report an effect of marijuana use on the risk of head and neck cancer. Not only did we find an elevated cancer risk among marijuana users, but we also observed dose-response associations for frequency and years of marijuana use, adjusting for several potential confounders.

Marijuana use in the United States increased dramatically among teenagers and young adults in the mid-to-late 1960s, *i.e.*, among persons born between 1941 and 1955. Assuming marijuana use is associated with cancer risk with an induction/latency period of 20–30 years, this cohort will be the earliest possible group to experience and clinically manifest elevated risks of head and neck cancer. This suggests that observed risks should be greater among subjects younger than 55 years. Our analyses, restricted to the younger population (<55 years old) with only 32% of our cases (n = 55) and 36% of controls (n = 63) suggested a stronger marijuana effect in the subpopulation of younger subjects than in the population as a whole. The dose-response relationships were also stronger in younger subjects. No association was observed for subjects 55 years or older.

Others have speculated that the uniquely characteristic technique of smoking marijuana might influence the tumor site of development (19 , 20) . The more rapid and deeper inhalation technique of marijuana smoking may lead to earlier and more pronounced deposition of carcinogens in the particulate phase of the smoke at relatively narrow sites in the upper airway, such as the larynx, as well as in the central portions of the tracheobronchial tree, because of turbulence and inertial impaction (11 , 37) . At the same time, the prolonged inhalation time might permit larger particles in the tar phase to deposit in the oral cavity, especially on the tongue. Because of the limited sample size, we would not be able to analyze marijuana use and head and neck cancer stratified by tumor site. Future studies with larger sample size are warranted to explore this aspect.

Possible interaction effects were suggested between marijuana use and other risk factors for head and neck cancer. The interplay between carcinogens and intrinsic host susceptibility is an important factor in environmental carcinogenesis. Mutagen hypersensitivity, an indirect marker for DNA repair, interacts with tobacco smoking in head and neck cancer risk (38, 39, 40, 41) . Synergy between mutagen hypersensitivity and marijuana use was suggested in this study because the effects were more than additive, which suggests that the development of the upper aerodigestive cancers may be affected by gene-environment interaction. Synergy (greater than additive effects) was also suggested between

marijuana use and tobacco smoking. These results suggest that the carcinogenic properties of marijuana may include not only the carcinogens present in tobacco but also other potential carcinogens and/or other factors that might particularly predispose marijuana smokers to cancer development, such as the Δ9-tetrahydrocannabinol-related impairment of antitumor immunity (12) . Because of the low power for testing these interactions, however, the present findings will need to be replicated in future studies.

In summary, this is the first epidemiological report that marijuana smoking is associated with a dose-dependent increased risk of head and neck cancer. This association is supported by a series of case reports and by experimental studies that provide a biologically plausible basis for the hypothesis that marijuana is a risk factor for human head and neck cancer. Given the long induction/latency period of head and neck cancer and the first wave of marijuana use in the 1960s in the United States, it is now time to examine the association between marijuana use and cancer risk. Large epidemiological studies are needed to replicate our results, to examine the relationships between marijuana use and increased risk of cancer, and to assess potential interactions between marijuana use and other risk factors.

Footnotes

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[3] The abbreviations used are: OR, odds ratio; CI, confidence interval.

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Δ-9-Tetrahydrocannabinol Enhances Breast Cancer Growth and Metastasis by Suppression of the Antitumor Immune Response[1]

Robert J. McKallip,[2]* Mitzi Nagarkatti,* and Prakash S. Nagarkatti†

In the current study, we tested the central hypothesis that exposure to Δ-9-tetrahydrocannabinol (Δ^9-THC), the major psychoactive component in marijuana, can lead to enhanced growth of tumors that express low to undetectable levels of cannabinoid receptors by specifically suppressing the antitumor immune response. We demonstrated that the human breast cancer cell lines MCF-7 and MDA-MB-231 and the mouse mammary carcinoma 4T1 express low to undetectable levels of cannabinoid receptors, CB1 and CB2, and that these cells are resistant to Δ^9-THC-induced cytotoxicity. Furthermore, exposure of mice to Δ^9-THC led to significantly elevated 4T1 tumor growth and metastasis due to inhibition of the specific antitumor immune response in vivo. The suppression of the antitumor immune response was mediated primarily through CB2 as opposed to CB1. Furthermore, exposure to Δ^9-THC led to increased production of IL-4 and IL-10, suggesting that Δ^9-THC exposure may specifically suppress the cell-mediated Th1 response by enhancing Th2-associated cytokines. This possibility was further supported by microarray data demonstrating the up-regulation of a number of Th2-related genes and the down-regulation of a number of Th1-related genes following exposure to Δ^9-THC. Finally, injection of anti-IL-4 and anti-IL-10 mAbs led to a partial reversal of the Δ^9-THC-induced suppression of the immune response to 4T1. Such findings suggest that marijuana exposure either recreationally or medicinally may increase the susceptibility to and/or incidence of breast cancer as well as other cancers that do not express cannabinoid receptors and are resistant to Δ^9-THC-induced apoptosis. *The Journal of Immunology*, **2005, 174: 3281–3289.**

Marijuana is one of the most common drugs of abuse and it medicinal use is the subject of current debate. Δ-9-tetrahydrocannabinol (Δ^9-THC),[3] the major psychoactive component in marijuana (1), and other synthetic cannabinoids have been used as potential therapeutic agents in alleviating such complications as intraocular pressure in glaucoma, cachexia, nausea, and pain (2). Interest in the potential medicinal use of cannabinoids grew with the discovery of two cannabinoid receptors, CB1 and CB2 (3, 4). CB1 is predominantly expressed in the brain, whereas CB2 is primarily found in the cells of the immune system (1, 4). Furthermore, endogenous ligands for these receptors capable of mimicking the pharmacological actions of Δ^9-THC have also been discovered. Such ligands were designated endocannabinoids and include anandamide and 2-arachidonoyl glycerol (5–7). The physiological function of endocannabinoids and cannabinoid receptors remains unclear. Recent work from our laboratory and others suggest that cannabinoids, including Δ^9-THC, may be effective in treating a variety of cancers including lymphomas, leukemias, and gliomas (8–10).

In contrast to these potentially beneficial properties, the use of marijuana has been associated with unwanted effects such as increased susceptibility to infections (11–13), and increased incidence of head and neck cancers in humans (14) and lung cancer in mice (15). Δ^9-THC possesses significant immunomodulatory properties. For example, exposure of macrophages to Δ^9-THC led to decreased production of TNF-α and NO in response to LPS (16). Additionally, exposure of macrophages to Δ^9-THC caused an impairment of their Ag-presenting capabilities (17). Exposure to cannabinoids can also lead to significant reductions in the proliferative and cytolytic response of T lymphocytes and Ab production by B cells (18–21). In addition, other studies conducted in vivo have shown that exposure to Δ^9-THC can lead to increased susceptibility to infections with various pathogens including *Herpes simplex* and Friend leukemia virus (12, 13). Furthermore, exposure to Δ^9-THC has been shown to suppress the immune response to lung cancers in mice (15).

Both the innate and adaptive immune responses are believed to be involved in controlling the growth of many cancers. Coordination of the two arms of the immune system is largely controlled by cytokines produced by cells such as dendritic cells. In addition, T regulatory cells and NKT cells have been implicated in the control of the antitumor immune response (22–24). In general, it is believed that a Th1 response is necessary for an effective immune response to be mounted against most tumors (25). IL-2 and IFN-γ are two cytokines that promote a Th1 response, while IL-4 and IL-5 promote a Th2 response. In addition, a number of cytokines possess suppressive activity. For example, IL-10 has been shown to suppress the Th1 response. In previous work, it was demonstrated that exposure to Δ^9-THC could lead to alterations in cytokine production which resulted in suppression of the immune response to *Legionella pneumophila* (26) as well as a lung cancer cell line (15).

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[3] Abbreviations used in this paper: Δ^9-THC, Δ-9-tetrahydrocannabinol; SOCS, suppressor of cytokine signaling; MMTV, mouse mammary tumor virus.

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Current work examining the potential use of Δ^9-THC and other cannabinoids for the treatment of cancers relies on the expression of CB1 and/or CB2 by the targeted tumor. However, little is known about the effect of Δ^9-THC exposure on the generation, growth, or response to tumors with low to no expression of CB1 and/or CB2. Because CB1 and CB2 are primarily expressed by tumor of neural and immune origins, respectively, it is possible that the majority of tumors originating in other tissues would be significantly less sensitive to Δ^9-THC-mediated killing and because Δ^9-THC is highly immunosuppressive, such tumors may find a favorable environment for growth and progression.

The immune system is suggested to play a key role in controlling the development of cancers as suggested by the findings that immunosuppressed individuals are at a higher risk for developing cancer. For example, there is an increased incidence of Karposi sarcoma, non-Hodgkins lymphoma, Burkitt lymphoma, and cervical cancer in AIDS patients (27). In addition, there have been reports of increased susceptibility to various lymphomas and cutaneous neoplasms following organ transplantation (28–30). Interestingly, there is evidence that de novo breast cancer incidence may increase following liver transplantation (31), suggesting the possibility that the immune system can play an important role in the development of this type of cancer. Therefore, in the current study, using a breast cancer model, we examined the effect of Δ^9-THC exposure on the immune response to and the growth of cancer cells that expresses low to undetectable levels of cannabinoid receptors.

Materials and Methods

Mice

Adult female BALB/c mice were purchased from the National Institutes of Health. SCID-NOD mice were purchased from The Jackson Laboratory. The mice were housed in polyethylene cages and given rodent chow and water ad libitum. Mice were housed in rooms maintaining a temperature of 74 ± 2°F and on a 12-h light/dark cycle.

Reagents

Δ^9-THC was obtained from the National Institute on Drug Abuse and was initially dissolved in DMSO (Sigma-Aldrich) to a concentration of 20 mM and stored at −20°C. Δ^9-THC was further diluted with tissue culture medium for in vitro studies and PBS for in vivo studies. SR141716A and SR144528 were obtained from Sanofi Recherche. Anti-IL-4 mAbs (11B.11) were obtained from the Biological Resources Branch, National Cancer Institute-Frederick Cancer Research and Development Center. Anti-IL-10 mAbs were obtained from BD Pharmingen.

Cell lines

The murine mammary cell carcinomas 4T1 and EMT6 syngeneic to BALB/c mice, the human breast cancer cell lines, MCF-7 and MDA-MB-231, the human T lymphoblastic leukemia cell line, Jurkat, and the human glioma U87 were maintained in RPMI 1640 (Life Technologies Laboratories) supplemented with 5% FCS, 10 mM HEPES, 1 mM glutamine, 40 μg/ml gentamicin sulfate, and 50 μM 2-ME.

RNA isolation and RT-PCR

RNA was isolated from ~1×10^7 cells using the RNeasy Mini kit (Qiagen). As CB1 and CB2 are encoded by single exons, a DNase digestion was included in the isolation procedure to limit the possibility of PCR amplification of CB1 and CB2 from genomic DNA. cDNA was prepared with the Qiagen OmniScript RT kit using 1 μg of RNA as template for first strand synthesis. Mouse and human CB1 was amplified using primers H CB1 U (5′-CGTGGGCAGCCTGTTCCTCA-3′) and H CB1 L (5′-CATGCGGGCTTGGTCTGG-3′), which yield a product of 403 bp. Human CB2 was amplified using primers H CB2 U (5′-CGCCGGAAGCCCTCATACC-3′) and H CB2 L (5′-CCTCATTCGGGCCATTCCTG-3′), which yield a product of 522 bp. Mouse CB2 was amplified using M CB2 (5′-CCGGAAAAGAGGATGGCAATGAAT-3′) and M CB2 (5′-CTGCTGAGCGCCCTGGAGAAC-3′) which yields a product of 479 bp. β-Actin was used as a positive control (primers M BA U (5′-AAGGCCAACCGTGAAAAGATGACC-3′) and M BA L (5′-ACCGCTCGTTGCCAATAGTGATGA-3′), product size of 427 bp). PCR were conducted using the following parameters: 95°C for 15 s, 58°C for 15 s, and 72°C for 30 s for 35 cycles, followed by a final 5 min at 72°C in an Applied Biosystems GeneAmp 9700. The resulting PCR products were separated on a 1% agarose gel.

Detection of Δ^9-THC-mediated cell death in vitro

Tumor cells or splenocytes (1×10^6 cells/well) were cultured in 24-well plates in the presence or absence of various concentrations of Δ^9-THC for 24 h. Next, the cells were harvested, washed twice in PBS and analyzed for cell viability by trypan blue dye exclusion.

Quantification of the effect of Δ^9-THC exposure on 4T1 tumor growth and metastasis in vivo

Groups of BALB/c or SCID-NOD mice were injected s.c. with 3×10^5 4T1 tumor cells. Three days later, the mice then were exposed every other day for ~3 wk to various doses of Δ^9-THC (12.5, 25, or 50 mg/kg body weight) or vehicle (DMSO) control. The tumor volume was observed, recorded, and calculated using the following equation: tumor volume = length × width^2 × 0.52. In addition, the level of metastasis was determined by directly quantifying the number of metastatic nodules located in the lungs, by H&E staining of lung sections, and by assessing tumor burden by determining the increase in lung weight.

In vivo antitumor immune response

BALB/c mice were first sensitized to 4T1 by injecting them i.p. with 1×10^6 irradiated 4T1 tumor cells twice at 2-wk intervals. Two weeks following the final sensitization, the mice were injected s.c. into their rear footpads with 1×10^5 irradiated 4T1 tumor cells or 1×10^5 irradiated EMT6 tumor cells (negative control). Groups of mice were then treated i.p. with various doses of Δ^9-THC (0, 12.5, 25, or 50 mg/kg) daily for 4 days. Four days following the challenge with the irradiated tumor cells the immune response was determined by aseptically removing the draining lymph node and quantifying the increase in lymph node mass, cell number, and lymphocyte DNA synthesis. In experiments examining the role of anti-IL-4 mAbs and anti-IL-10 mAbs, mice received a single injection of 5 mg or 5 mg/kg of mAbs, respectively, which was previously shown to effectively reduce IL-4 and IL-10 concentrations (15, 32).

In vitro proliferation assay

The spleens and lymph nodes from control or Δ^9-THC-treated mice were placed into 10 ml of RPMI 1640 (Life Technologies Laboratories) supplemented with 10% FCS, 10 mM HEPES, 1 mM glutamine, 40 μg/ml gentamicin sulfate, and 50 μM 2-ME, referred to as complete medium. The spleens and lymph nodes were prepared into a single cell suspension using a laboratory homogenizer, washed twice, and adjusted to 5×10^6/ml in complete medium. The splenocytes and lymph node cells (5×10^5 in 100 μl/well) were cultured in 96-well flat-bottom plates and stimulated with various concentrations of irradiated 4T1 tumor cells for 4 days. During the final 8 h of culture, the cells were pulsed with 2 μCi of [^{3}H]thymidine. DNA synthesis was determined by beta scintillation counting (33, 34).

Cytokine detection

BALB/c mice were first sensitized to 4T1 by injecting them i.p. with 1×10^6 irradiated 4T1 tumor cells twice at 2-wk intervals. Two weeks following the final sensitization injection the mice were injected s.c. into their rear footpads with 1×10^5 irradiated 4T1 tumor cells. Groups of mice were then treated daily for 4 days with vehicle control or Δ^9-THC (50 mg/kg i.p.). Four days following the challenge with the irradiated tumor cells the draining lymph node were removed and adjusted to 2.5×10^6/ml in RPMI 1640 containing 10% FCS. The lymph node cells were cultured in a 96-well flat-bottom plate (200 μl/well) for 24 h, after which the levels of IFN-γ, TGF-β, IL-4, IL-10, and TNF were determined using the methods described in the Quantikine M ELISA kits (R&D Systems).

Microarray analysis of gene expression

Total RNA was isolated from lymph node cells isolated from 4T1-immunized mice that were stimulated in their rear footpads with irradiated 4T1 tumor cells (1×10^5 s.c.) and treated i.p. for 4 days with vehicle or 50 mg/kg Δ^9-THC using the RNeasy Mini kit (Qiagen). Labeled cDNA probes were synthesized from the RNA samples using the Ampolabeling-LPR kit (SuperArray). The labeled cDNA probes were hybridized to individual GEArray Q series mouse Th1, Th2, Th3 array membranes overnight at 60°C with continuous agitation at 5–10 rpm. The membranes were washed twice for 10 min at 60°C with 2× SSC, 1% SDS solution, and twice for 10

min at 60°C with 0.1× SSC, 0.5% SDS. Nonspecific binding was blocked by incubating the membranes with GEAblocking solution for 40 min. The membranes were labeled with alkaline phosphatase-conjugated streptavidin alkaline phosphatase for 10 min. Excess alkaline phosphatase was removed by washing the membranes four times with Buffer F (SuperArray) for 5 min and rinsing the membranes with Buffer G. Gene expression was detected using CDP-Star chemiluminescent substrate and exposing the membranes to x-ray film. The data were analyzed by converting the x-ray image into a grayscale TIFF file and using the ScanAlzye software program to convert the data into numerical data. Finally, data analysis was performed using the GEArray Analyzer data analysis software (SuperArray). Data was normalized using housekeeping genes including β-actin, GAPDH, cyclophilin A, and ribosomal protein L13a.

Statistical analysis

Student's *t* test or Tukey Kramer test was used to compare vehicle and Δ^9-THC-treated groups. $p < 0.05$ was considered to be statistically significant.

Results

Expression of CB1 and CB2 in human and murine breast cancer cells

The expression of CB1 and CB2 mRNA was determined by RT-PCR. The results showed that splenocytes expressed both receptors, while in the 4T1 breast cancer cells, CB1 and CB2, mRNA was not detectable (Fig. 1*A*). Similar results were seen when we examined the expression of CB1 and CB2 in the human breast cancer cell lines MCF-7 and MDA-MB-231 (Fig. 1*C*). In this experiment, Jurkat cells were used as a positive control for CB2 expression and the human glioma U87 was used as a positive control for CB1 expression. The results demonstrated that in both human breast cancer cell lines there was very low detectable expression of CB1 while CB2 expression was not detected.

Sensitivity of 4T1 and MCF-7 to Δ^9-THC-induced cell death

Next we examined whether 4T1 or MCF-7 were sensitive to Δ^9-THC-induced cytotoxicity compared with other cells reported to be sensitive to Δ^9-THC. To this end, 4T1 breast cancer cells and splenocytes from BALB/c mice were cultured for 24 h in RPMI 1640 containing 5% FCS in the presence of various concentrations of Δ^9-THC (0, 5, 10, or 20 μM). The viable cell number was determined by trypan blue dye exclusion (Fig. 1*B*). The results demonstrated that although the splenocytes were highly sensitive to Δ^9-THC-induced killing, the 4T1 cells were relatively resistant. No decrease in viable cell number in the 4T1 breast cancer cells was observed even at the highest concentration of Δ^9-THC tested. In contrast, splenic culture showed a significant reduction in viable cell number following exposure to concentration of Δ^9-THC as low as 5 μM. In addition, we examined whether the human breast cancer cell line MCF-7 was sensitive to Δ^9-THC-mediated cell death. To this end, MCF-7 and Jurkat cells were cultured for 24 h in RPMI 1640 containing 5% FCS in the presence of various concentrations of Δ^9-THC (0, 5, 10, or 20 μM) and the viable cell number was determined by trypan blue dye exclusion (Fig. 1*D*). The results showed that while the Jurkat cells were sensitive to Δ^9-THC-mediated killing at concentrations as low as 5 μM, the MCF-7 cells were resistant to Δ^9-THC-induced toxicity. Together, these data suggested that both the murine 4T1 and the human MCF-7 human breast cancer cell lines are resistant to killing mediated by Δ^9-THC exposure.

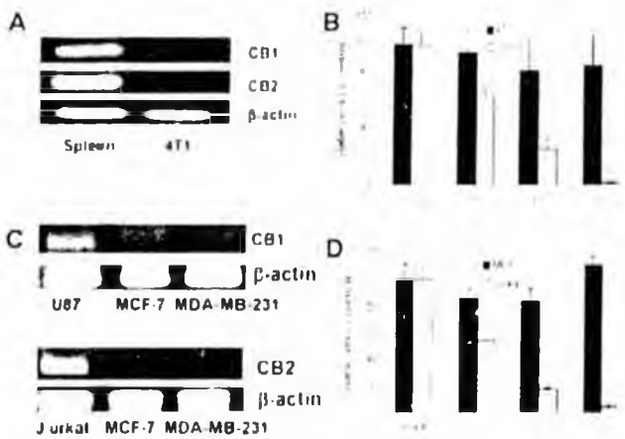

FIGURE 1. Human and murine breast cancer cells expression of CB1 and CB2 and sensitivity to Δ^9-THC-induced cytotoxicity. The expression of CB1 and CB2 mRNA was determined by RT-PCR analysis. Total RNA was isolated from mouse spleen cells, 4T1, Jurkat, MCF-7, MDA-MB-231, and U87 tumor cells. mRNA was reverse transcribed and amplified by PCR with primers specific for CB1, CB2, and β-actin. A photograph of ethidium bromide-stained amplicons is depicted (*A* and *C*). The effect of Δ^9-THC on cell viability was determined by culturing the cells with various concentrations of Δ^9-THC for 24 h in medium containing 5% FCS. The cell viability was determined by trypan blue dye exclusion. The data were expressed as percent of control viable cell number (*B* and *D*).

Δ^9-THC-exposure leads to increased growth of the 4T1 breast cancer in vivo

Next, we examined whether exposure to Δ^9-THC had any effect on the local growth of the 4T1 tumor (Fig. 2*A*). To this end, BALB/c mice were injected s.c. with 3×10^5 4T1 tumor cells. Three days following the tumor injection, the mice were exposed every other day for 18–21 days to either vehicle or various doses of Δ^9-THC (12.5, 25, or 50 mg/kg). Tumor growth was monitored and the data revealed that exposure to 25 mg/kg Δ^9-THC led to a significant increase in tumor mass. This effect was even more pronounced in mice treated with 50 mg/kg Δ^9-THC.

Δ^9-THC-exposure leads to increased metastasis of 4T1 tumor to the lung

In addition to examining the effects of Δ^9-THC on the local growth of the 4T1 tumor, we examined whether exposure to Δ^9-THC would have any effect on the level of metastasis in the lungs. To this end, BALB/c mice were injected s.c. with 3×10^5 4T1 tumor cells. Three days following the tumor injection, the mice were exposed every other day to either vehicle control or various doses of Δ^9-THC (12.5, 25, or 50 mg/kg). The lungs from the tumor-bearing mice were harvested 18–21 days following tumor injection and the level of metastasis was quantified (Fig. 2, *B* and *C*). The results showed that exposure to 25 or 50 mg/kg Δ^9-THC led to a significant increase in the number of tumor nodules located in the lungs. H&E staining of lung sections revealed that Δ^9-THC-treatment led to a dose-dependent increase in the size of the metastatic nodules (Fig. 2*D*). In addition, tumor burden in the lungs was quantified by determining the increase in lung mass in tumor bearing mice vs control mice and the results show that Δ^9-THC-treatment led to a significant increase in lung mass (Fig. 2*E*). Together, these results suggested that Δ^9-THC-exposure increased the metastasis of 4T1 tumor to the lungs.

The effect of Δ^9-THC exposure on 4T1 tumor growth in SCID-NOD mice

Next, the role of the immune system in the observed increase in 4T1 tumor growth and metastasis following Δ^9-THC was evaluated using the SCID-NOD model. SCID-NOD mice are devoid of an antitumor immune response. Therefore, any effect of Δ^9-THC

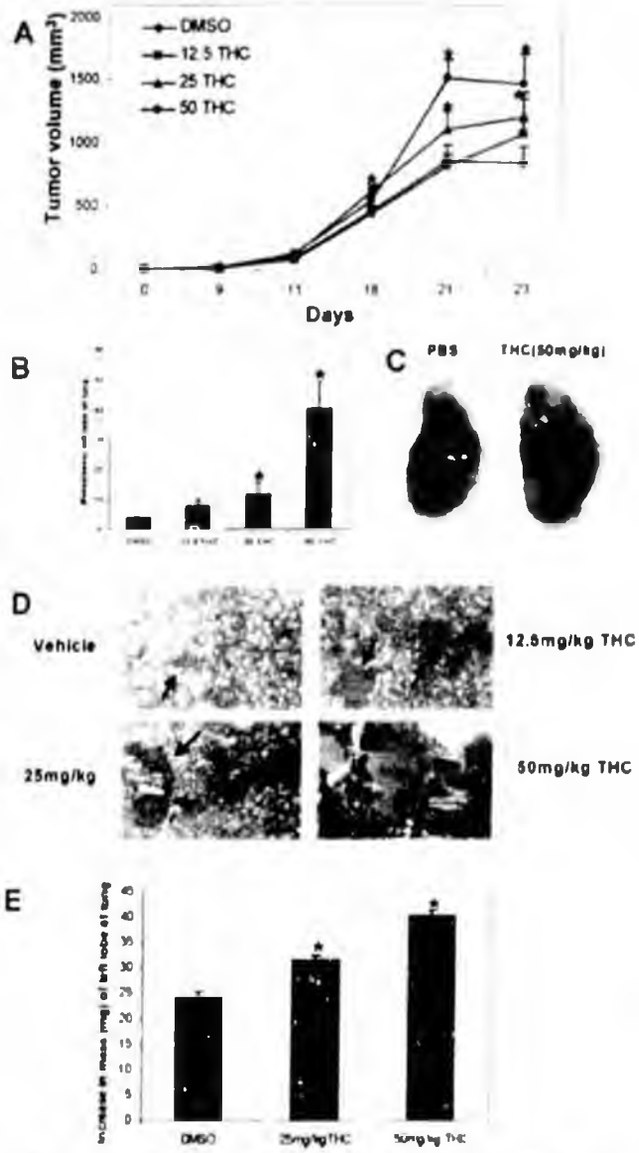

FIGURE 2. Δ⁹-THC exposure leads to an increase in number and size of 4T1 tumors metastasizing to the lungs in vivo. Mice injected s.c. with 3×10^5 4T1 tumor cells were treated with various concentration of Δ⁹-THC every other day for 21 days. Local tumor volume was determined (*A*). The lungs were harvested and the metastases were quantified (*B* and *C*). Sections of the lungs were stained with H&E (*D*). The arrows indicate the sites of tumor growth. •, Statistically significant difference ($p < 0.05$) when compared with the controls. The results are representative data of experimental groups containing four mice. The experiment has been repeated three times with similar results. Tumor burden was quantified by determining the increase in the weight of the lungs from tumor bearing mice compared with control mice (*E*).

on tumor growth in these mice would be independent of an effect on the immune response. To this end, SCID-NOD mice were injected s.c. with 4T1 tumor cells. The mice were then treated with the vehicle or 25 mg/kg Δ⁹-THC every other day for 19 days. Local tumor growth and metastasis were recorded. The results revealed that Δ⁹-THC exposure did not result in a significant increase in tumor growth (Fig. 3*A*) or metastasis (Fig. 3*B*), suggesting that the effects of Δ⁹-THC on the growth of the 4T1 tumor in immunocompetent mice may be directly related to an effect on the immune system.

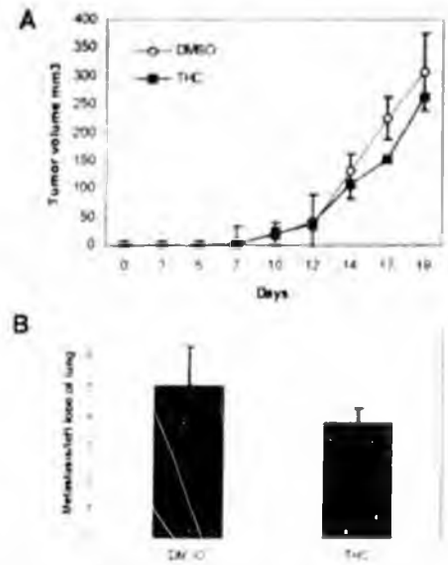

FIGURE 3. The effect of Δ⁹-THC exposure on 4T1 tumor growth in SCID-NOD mice. SCID-NOD mice were injected s.c. with 3×10^5 4T1 tumor cells. The mice were then treated i.p. with the vehicle control or 25 mg/kg Δ⁹-THC every other day for 19 days. Local tumor growth (*A*) and metastasis were recorded (*B*). The results are representative data of experimental groups containing four mice. The experiment was repeated three times with similar results.

Δ⁹-THC exposure directly suppresses the immune response to 4T1 in vivo

To directly examine the effect of Δ⁹-THC exposure on the antitumor immune response, we used a modified version of the popliteal lymph node assay. More specifically, mice were first sensitized to 4T1 by injecting them twice, separated by 2 wk, with irradiated 4T1 tumor cells. Two weeks following the final sensitization injection, the mice were rechallenged in their rear footpads with irradiated 4T1 tumor cells and either received daily i.p. injections of Δ⁹-THC (25 or 50 mg/kg) or vehicle. The immune response was determined 4 days following rechallenge by harvesting the lymph nodes, draining the site of tumor injection, and assessing the increase in lymph node mass (Fig. 4*A*) and cell number (Fig. 4*B*) compared with the same lymph nodes from mice not receiving the rechallenge. The results showed that rechallenge with 4T1 led to a significant and measurable immune response and that exposure to Δ⁹-THC at concentrations as low as 25 mg/kg significantly suppressed the antitumor immune response against 4T1. In addition, groups of mice sensitized against 4T1 received a challenge with an unrelated syngeneic mammary carcinoma (EMT6), and such mice showed no significant immune response (data not shown), demonstrating that the immune response in the sensitized mice was specific for 4T1. Next, the effect of Δ⁹-THC exposure on the growth and metastasis of 4T1 tumor cells in 4T1-sensitized mice was examined and the data showed that exposure to Δ⁹-THC led to quicker appearance of detectable tumors (Fig. 4*C*), an increase in tumor size (Fig. 4*D*), and an increase in the level of metastatic lesions in the lungs (Fig. 4*E*). Together, the results from these experiments demonstrated 4T1 tumor can be immunogenic and that exposure to Δ⁹-THC can suppress the immune response against 4T1 tumor, which may account for enhanced tumor growth and metastasis.

Δ⁹-THC exposure leads to suppression of the tumor-specific proliferative response

To further examine the effect of Δ⁹-THC on the antitumor immune response, we determined the effect of Δ⁹-THC exposure on the

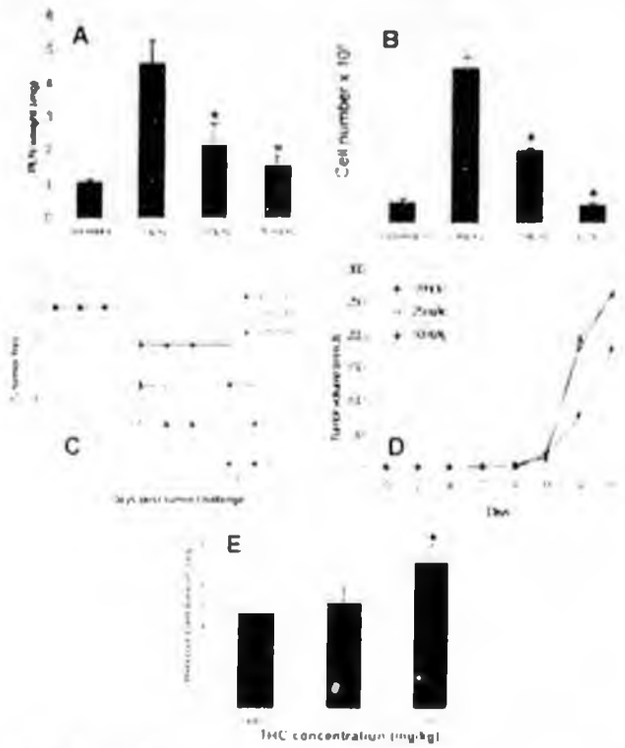

FIGURE 4. Δ^9-THC exposure directly suppresses the immune response to 4T1 in vivo. 4T1-sensitized mice were challenged s.c. in their footpads with irradiated 4T1 (1×10^5 cells). After which, the mice were treated daily for 4 days with CB1 (SR141716A), CB2 (SR144528) antagonists, or vehicle 1 h before exposure to Δ^9-THC (25 mg/kg). The immune response was assayed by determining the increase in lymph node mass (*A*) and cell number (*B*) compared with unchallenged mice. *, Statistically significant difference ($p < 0.05$) when compared with the controls. The effect of Δ^9-THC exposure on the growth and metastasis of 4T1 tumor cells in 4T1-sensitized mice was examined. To this end, 4T1-sensitized mice were injected s.c. with 1×10^5 live 4T1 cells. The mice were treated every other day for 16 days with vehicle or Δ^9-THC (25 or 50 mg/kg). The tumor incidence (*C*), tumor mass (*D*), and number of metastatic lesions in the lungs were determined (*E*). The results are representative data of experimental groups containing four mice. The experiment was repeated three times with similar results.

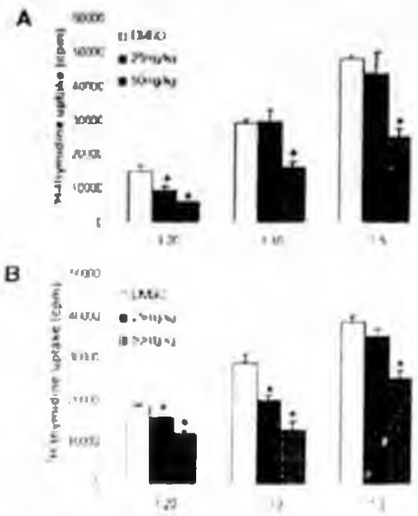

FIGURE 5. Δ^9-THC exposure leads to suppression of the tumor-specific proliferative response. 4T1-sensitized mice were treated for 4 days with vehicle or Δ^9-THC (25 or 50 mg/kg). Next, spleens (*A*) and lymph nodes (*B*) were harvested and cultured in vitro for 4 days with irradiated 4T1 tumor cells. Various responder (splenocytes or lymph node cells) to stimulator (irradiated 4T1 tumor cells) were tested. The proliferative response was determined by [^{3}H]thymidine uptake. The experiment was repeated three times with similar results. *, Statistically significant difference ($p < 0.05$) when compared with the controls.

proliferative response to 4T1. To this end, sensitized mice were treated for 4 days with various concentrations of Δ^9-THC (0, 25, and 50 mg/kg). Next, the splenocytes and lymph node cells were harvested and cultured in vitro for 4 days with irradiated 4T1 tumor cells. The proliferative response was determined by [^{3}H]thymidine uptake and the results revealed that in vivo exposure to Δ^9-THC led to a significant suppression of the proliferative response of splenocytes (Fig. 5*A*) and lymph nodes cells (Fig. 5*B*) to 4T1.

The effect of CB1 and CB2 antagonist on Δ^9-THC-induced suppression of the immune response to 4T1 in vivo

To investigate the role of CB1 and CB2 in Δ^9-THC-induced suppression of the antitumor immune response to 4T1, sensitized mice were challenged s.c. in their footpads with irradiated 4T1. Next, the mice were treated daily for 4 days with CB1 (SR141716A), CB2 (SR144528) antagonists (20 mg/kg), or vehicle 1 h before exposure to Δ^9-THC (25 mg/kg). The immune response was assayed by determining the increase in lymph node mass (Fig. 6*A*), cell number (Fig. 6*B*), and proliferation (Fig. 6*C*) compared with unchallenged mice. The results demonstrated that treatment with the CB2 antagonist, but not the CB1 antagonist, could significantly reverse the Δ^9-THC-induced suppression of the immune response against 4T1, suggesting a prominent role for CB2 in the observed Δ^9-THC-mediated suppression of the antitumor immune response.

Δ^9-THC exposure alters cytokine production

Previous reports have indicated that exposure to Δ^9-THC can alter the production of various cytokines (35, 36). Because the antitumor immune response is primarily mediated by Th1-directed immune response, we examined whether exposure to Δ^9-THC had any effect on the production of Th1 vs Th2 cytokines. To this end, 4T1-sensitized mice were challenged s.c. with irradiated 4T1 tumors and then received daily injection with various doses of Δ^9-THC (vehicle, 25, and 50 mg/kg). Four days following the challenge with 4T1, the lymph node cells draining the site of injection were harvested, counted, and cultured (1×10^6 cells/well) for 24 h in 96-well plates. Next, the supernatants were tested for the presence of various Th1 and Th2 cytokines (Fig. 7*A*). The results showed that exposure to 25 mg/kg Δ^9-THC led to a dramatic increase in the Th2 cytokines IL-4 and IL-10, suggesting that at this concentration, Δ^9-THC enhances the Th2 response. In addition, levels of IFN-γ were found to be elevated following exposure to Δ^9-THC. Interestingly, exposure to 50 mg/kg Δ^9-THC led to a significant reduction in IL-4, IFN-γ, and IL-10 compared with the vehicle or 25 mg/kg Δ^9-THC groups, suggesting the possibility that at a higher concentration, Δ^9-THC was leading to a more generalized suppression of the antitumor immune response, possibly due to the induction of apoptosis (21).

Anti-IL-4 and anti-IL-10 mAbs partially prevent Δ^9-THC-induced suppression of the immune response to 4T1

Next, we examined the effects of Abs against IL-4 or IL-10 on the Δ^9-THC-induced suppression of the immune response to 4T1. To this end, 4T1-sensitized mice were first challenged in their rear footpads with irradiated 4T1 cells. Groups of mice were then treated with vehicle + isotype control mAbs, Δ^9-THC (25 mg/kg/day) + isotype control mAbs, Δ^9-THC (25 mg/kg/day) + anti-

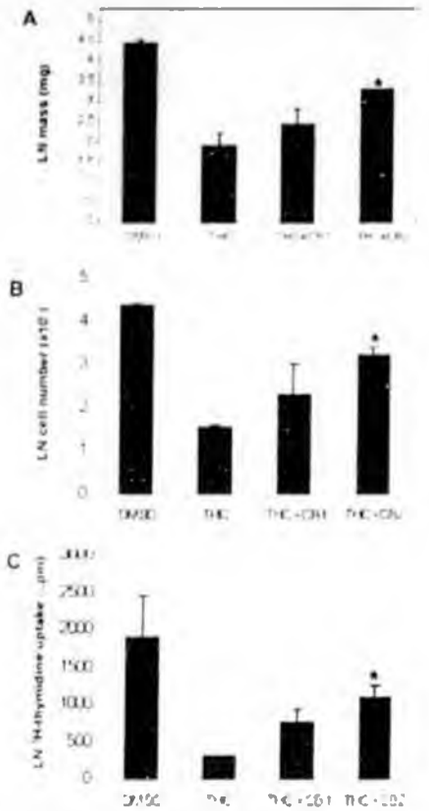

FIGURE 6. The effect of CB1 and CB2 antagonist on Δ⁹-THC-induced suppression of the immune response to 4T1 in vivo. 4T1-sensitized mice were challenged s.c. in their footpads with irradiated 1×10^5 irradiated 4T1 cells. Next, the mice were treated daily for 4 days with CB1 (SR141716A), CB2 (SR144528) antagonists (20 mg/kg), or vehicle 1 h before exposure to Δ⁹-THC (25 mg/kg). The immune response was assayed by determining the increase in lymph node mass (*A*), cell number (*B*), and DNA synthesis (*C*) compared with lymph nodes from unchallenged mice. The results are representative data of experimental groups containing four mice. The experiment was repeated three times with similar results. •, Statistically significant difference ($p < 0.05$) when compared with the controls.

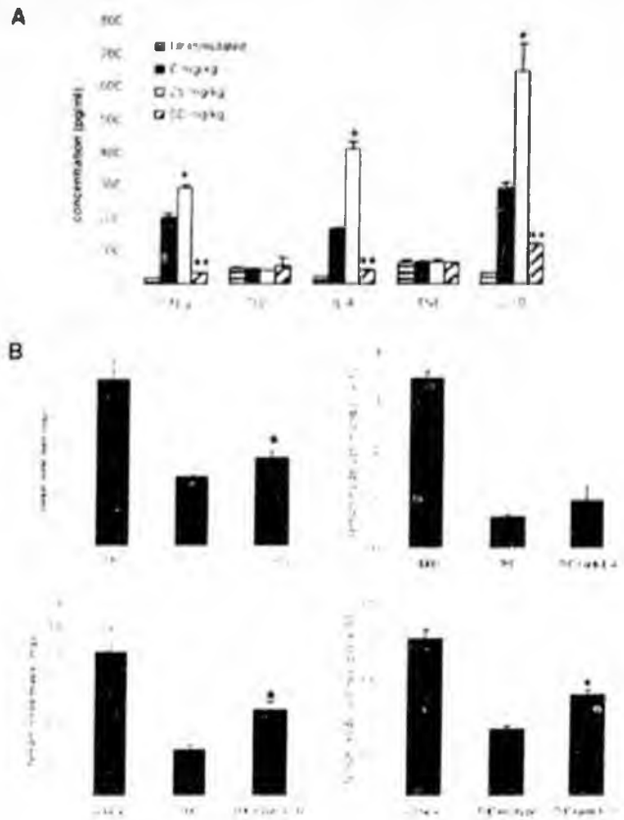

FIGURE 7. Δ⁹-THC exposure leads to alteration in cytokine production. 4T1-sensitized mice were challenged s.c. with 1×10^5 irradiated 4T1 cells and then received daily injection with various doses of Δ⁹-THC (vehicle, 25, and 50 mg/kg). Four days following the challenge with 4T1, the lymph node cells draining the site of injection were harvested, counted and cultured (1×10^6 cells/well) for 24 h in 96-well plates. Next, the supernatants were tested for the presence of various Th1 and Th2 cytokines (*A*). •, Statistically significant increase ($p < 0.05$) when compared with the untreated control. **, Statistically significant decrease ($p < 0.05$) when compared with the untreated control. To examine the effects of Abs against IL-4 or IL-10 on Δ⁹-THC-induced suppression of the immune response, 4T1-sensitized mice were first challenged in their rear footpads with irradiated 4T1 cells (1×10^5 cells). Groups of mice were then were treated with vehicle control + isotype control mAbs, Δ⁹-THC (25 mg/kg/day) + isotype control mAbs, Δ⁹-THC (25 mg/kg/day) + anti-IL-4 mAbs, or Δ⁹-THC (25 mg/kg/day) + anti-IL-10 mAbs. The immune response was assayed 4 days later by determining the mass and cell number of the lymph nodes draining the site of 4T1 injection (*B*). •, Statistically significant differences ($p < 0.05$) when compared with mice treated with Δ⁹-THC alone.

IL-4 mAbs, or Δ⁹-THC (25 mg/kg/day) + anti-IL-10 mAbs. The immune response was assayed 4 days later by determining the mass and cell number of the lymph nodes draining the site of 4T1 injection (Fig. 7*B*). The results showed that exposure to Δ⁹-THC led to a significant reduction in the lymph node mass. However, if the mice were treated with anti-IL-10 mAbs, or to a lesser extent, anti-IL-4 mAbs, the Δ⁹-THC-induced reduction in lymph node mass could be partially reversed. Together, these results further suggested a role for IL-4 and IL-10 in the Δ⁹-THC-induced suppression of the immune response to 4T1.

The effect of Δ⁹-THC exposure of gene expression in lymph node cells draining the site of 4T1 injection

Next, using cDNA array analysis, we screened for alterations in the expression of genes involved in the Th1 and Th2 response in lymph node cells draining the site of 4T1 injection following exposure of 4T1-sensitized mice to 25 mg/kg Δ⁹-THC. Of the 96 genes screened, the expression of 18 genes was significantly (>2-fold) altered in the lymph nodes cells from the Δ⁹-THC-treated mice. The expression of 6 genes was reduced, while the expression of 12 was increased in the lymph node cells isolated from the Δ⁹-THC-treated mice (Table 1). Included in the group of down-regulated genes were the Th1-associated genes, IL-1R, and the TNFR superfamily members DR6 and 4-1BB. In addition, the expression of a number of transcriptional regulators was reduced. Analysis of the genes that were up-regulated following exposure to Δ⁹-THC revealed several Th2-associated genes, including C2ta, eotaxin, IL-13R, IL-4, IL-4R, IL-5, GATA binding protein 3, and growth factor independent 1. In addition, the expression of a number of transcriptional regulators including, suppressor of cytokine signaling (SOCS)2, SOCS5, SOCS7, and Fos-like Ag 2 was increased. Taken together, the results from the cDNA analysis further suggested that exposure to Δ⁹-THC leads to suppression of genes regulating Th1 response and an increase in the Th2 response genes leading to an inefficient immune response against the 4T1 tumor in vivo.

Discussion

In the current study, we demonstrated that exposure to Δ⁹-THC can enhance the growth and metastasis of the 4T1 mammary

Table I. *cDNA array analysis of Th1/Th2-associated gene expression in 4T1-stimulated lymph node cells from vehicle- or Δ^9-THC-treated 4T1-sensitized mice*[a]

Gene Name	Description	Function	Accession Number	Fold Change[b] vs Vehicle
C2ta	Class II transactivator	Th2	NM_007575	+2.1
Scya11/eotaxin	Small chemokine ligand 11	Th2	U26426	+19.4
IL13RA2	Interleukin-13 receptor, α2	Th2	U65747	+4.4
IL-4	Interleukin-4	Th2	M25892	+4.8
IL-4ra	Interleukin-4 receptor, α	Th2	NM_010557	+4.4
IL-5	Interleukin-5	Th2	NM_010558	+2.9
GATA3	GATA binding protein 3	Th2	NM_008091	+2.9
Gfi1	Growth factor independent 1	Th2	NM_010278	+3.5
SOCS2	SOCS2	Trans. reg.	NM_007706	+2.0
SOCS5	SOCS5	Trans. reg.	NM_019654	+6.8
SOCS7	SOCS7	Trans. reg.	NM_080843	+2.6
Fosl2	Fos-like antigen 2	Trans. reg.	NM_008037	+3.2
IL-1R	Interleukin-1 receptor	Th1	U43673	−14.6
DR6	TNF receptor superfamily member	Th1	AF322069	−5.3
4-1BB	TNF receptor superfamily member	Th1	J04492	−5.7
Jund1	Jun proto-oncogene related gene d1	Trans. reg.	NM_010592	−11.7
JNKK2	MAP kinase kinase MKK7	Trans. reg.	U74463	−2.5
JNK1	Mitogen-activated protein kinase 8	Trans. reg.	AB005663	−2.9

[a] Summary of gene expression that was found to be increased or decreased in splenic cells isolated from Δ^9-THC-treated preimmunized mice following stimulation with irradiated 4T1 cells mice compared to the gene expression in splenic cells isolated from vehicle-treated preimmunized mice following stimulation with irradiated 4T1.

[b] Fold change represents the change in gene expression following normalization with β-actin gene expression.

[c] Trans. Reg., Transcriptional regulator.

carcinoma. This is in contrast to our previous finding in which we demonstrated that treatment with Δ^9-THC led to the elimination of the EL-4 leukemia in vivo (8). This stark contrast suggests that some tumors may be more resistant to Δ^9-THC-mediated killing and that the effects of Δ^9-THC on the immune system may play an important role in tumor growth and host survival in such tumor models. More specifically, we hypothesize that the degree of sensitivity of a tumor to Δ^9-THC may be directly related to the level of CB1 and CB2 expression. Importantly, these results would suggest that, although Δ^9-THC may be effective at killing tumors that express cannabinoid receptors, Δ^9-THC-exposure may actually lead to increased growth and metastasis of tumors with low to no expression of cannabinoid receptors due to suppression of the antitumor immune response.

The use of cannabinoids for the treatment of a number of cancers is currently under investigation (8, 10, 37, 38). However, little is known about the relationship between the level of cannabinoid expression and the sensitivity to Δ^9-THC killing. In the current study, we proposed that tumors that express little to no cannabinoid receptors would be relatively resistant to the cytotoxic effects of Δ^9-THC. This was shown in both a mouse and human breast cancer cell line. However, previous studies have shown that exposure to cannabinoids can lead to a decrease in the growth of some breast cancer cell lines in vitro. For example, exposure to anandamide inhibited the proliferation of the MCF-7 and EFM-19 human breast cancer cell line in vitro (38). It should be noted that although the use of CB1 antagonists led to the partial reversal of the anandamide-induced suppression of the proliferation of the EFM-19 cell line, the expression of CB1 or CB2 was not directly examined. To date, little has been reported about the expression and/or role of cannabinoid or vanilloid receptors in either human or mouse breast cancer cell lines. In this report, we demonstrated that the human breast cancer cell lines MCF-7 and MDA-MB-231 express only low levels of CB1 and undetectable levels of CB2 and that neither receptor was detectable in the mouse 4T1 mammary cell carcinoma. In addition, we demonstrated that 4T1 cells express high levels of vanilloid receptor 1 (data not shown). Therefore, because anandamide is also know to act as potent agonist for the vanilloid receptor 1 (39–41), it is possible that the breast cancer cells may be more sensitive to anandamide compared with Δ^9-THC due to the expression of vanilloid receptors. In addition, most previous studies did not directly examine the effects of anandamide on the growth of tumors in an in vivo setting. Therefore, depending on the role of the immune system in the control of growth of the specific tumor tested, it is still possible that the antitumor effects of anandamide and other cannabinoids may be offset by their immunosuppressive properties, ultimately leading to increased tumor growth as seen in our study.

In the current study, we used doses of Δ^9-THC up to 50 mg/kg. Importantly, there is evidence to suggest that the doses of Δ^9-THC used in the current study are pharmacologically relevant. Azorlosa et al. showed that levels as high as 1 μM could be obtained in the plasma of humans (42), and in separate report it was shown that Δ^9-THC can be concentrated 15- to 20-fold in some tissues (43). Therefore, it might be possible to reach levels as high as 20 μM in some tissues after recreational use. In an earlier study, Chan et al. showed that rats injected with 50 mg/kg body weight of Δ^9-THC led to a serum concentration of 10 μM of Δ^9-THC within 10 h of administration (44). Moreover, it has been proposed that the use of higher doses may be necessary in order for Δ^9-THC to be effective medicinally. Therefore, use of up to 50 mg/kg of Δ^9-THC should lead to physiologically relevant concentrations that correlate to the potential concentrations following recreational use and may also correlate with the concentrations necessary for some of the proposed clinical uses.

The immune response to tumors is believed to be mediated primarily by the Th1 response. Skewing of the immune response from the cell-mediated Th1 response to the humoral-mediated Th2 response may lead to a positive environment for tumor growth and development. In the current study, we showed that exposure to Δ^9-THC led to increased production of IL-4 and IL-10, and importantly, administration of Abs against these cytokines reversed the Δ^9-THC-mediated suppression of antitumor immunity. Increased levels of these cytokines have been associated with a number of cancers. For example, increased levels of IL-4 and IL-10 have been reported in patients with breast cancer and this was directly correlated to suppression of the immune response (45). In a separate study examining the immune response in patients with

breast and lung cancer, a shift toward the Th2 immune response was observed (46). Furthermore, increased levels of IL-10 secreting T-regulatory cells have been associated with the inability to mount an effective immune response to Hodgkins lymphoma (47). These studies highlight the potential involvement of the immune system in the development and progression of various tumors, including breast cancer, and suggest that skewing of the immune response to the Th2 phenotype may enhance the tumor's chances of survival. Therefore, the induction of a Th2 response following Δ^9-THC exposure may significantly increase tumor cell survival and ultimately facilitate tumor growth. Interestingly, in this study we also observed an increase in IFN-γ following Δ^9-THC exposure. This may suggest that, in the current study, Δ^9-THC led to an incomplete Th2 skewing of the response as seen in other tumor models (48) or to the activation of cells such as NKT or T regulatory cells (49, 50).

A number of other reports suggest that exposure to cannabinoids may affect the immune system by altering cytokine production in mice (35). For example, exposure to Δ^9-THC leads to inhibition of the Th1 response following *L. pneumophila* infection (26). Exposure of mice to cannabinoids in the concanavalin A-induced hepatitis model led to increased production of Th2-associated cytokines IL-10 and IL-6 and a reduction in the Th1-associated cytokines IL-2 and IFN-γ (51). Similar results were seen when examining the immune response to a murine lung cancer in which it was shown that the Δ^9-THC-induced suppression of the antitumor immune response was due to a Δ^9-THC-mediated shifting of cytokine production (15). Also, a recent study demonstrated that individuals who smoked marijuana on an occasional (eventual to monthly use) or regular basis (weekly to daily use) had abnormal T cell and NK cell functions and increased levels of TGF-β and IL-10 (52), suggesting a possible Th2 bias in humans, similar to what we reported in the current study. In addition, previous studies from our laboratory have shown that Δ^9-THC at doses of 50 mg/kg can lead to the induction of apoptosis in the thymus and spleen of naive mice. Previously, we demonstrated that concanavalin A-activated splenocytes and LPS-activated dendritic cells are relatively resistant to Δ^9-THC-induced apoptosis when compared with their naive counterparts and that the sensitivity correlated with the level of cannabinoid receptor expression (21, 53). Little is know about the expression of cannabinoid receptors in cells involved in the immune response to tumors or the effect of Δ^9-THC on their viability. Therefore, it is possible that Δ^9-THC may suppress the tumor-specific immune response by inducing apoptosis in Th1-associated cells reacting to the tumor challenge, resulting in the observed shift to the Th2 response.

Work using the 4T1 has shown that the immune response to this tumor is primarily mediated by $CD8^+$ cells (54). Additional studies suggested that NKT cells may play a negative role in the response to this tumor (55). For example, $CD1d^{-/-}$ mice had a significantly elevated response to the 4T1 tumor in vivo (55). Following stimulation, NKT can rapidly produce large quantities of IL-4 and IL-10 and have been implicated as possible negative or positive regulators of the antitumor immune response. Another cell that may play an important role in controlling the immune response is the $CD4^+CD25^+$ regulatory T cell. Interestingly, $CD4^+CD25^+$ regulatory T cells have been reported to suppress the antitumor immune response and this suppression was associated with the increased production of IL-10 (47, 56). To date, little is known about the effect of cannabinoids on NKT or $CD4^+CD25^+$ regulatory T cell functions. However, it is possible that Δ^9-THC exposure may directly lead to altered NKT and/or $CD4^+CD25^+$ regulatory T cell activity, resulting in the observed suppression of the antitumor immune response. In addition, it is possible that the observed suppression of the tumor-specific immune response may be mediated through alterations in dendritic cell function. This possibility is supported by work from our laboratory in which we demonstrated that dendritic cells are sensitive to Δ^9-THC-mediated apoptosis (53). The exact role of these cells in the Δ^9-THC-induced suppression of the antitumor immune response is currently being investigated in our laboratory.

Although, the importance of the immune system in protection against many of the common epithelial cancers remains controversial, it is becoming clear that the immune system plays a considerable role in the protection against virally induced or virus-associated tumors. For example, there is an increased rate of Karposi sarcoma, non-Hodgkins lymphoma, Burkitt lymphoma, and cervical cancer in AIDS patients (27). In addition, there have been reports of increased incidences of various lymphomas, cutaneous neoplasms, and de novo breast cancers following organ transplantation (28–31). Although, the immune response to 4T1 has not been fully elucidated, it has been postulated that the immune response may be directed against mouse mammary tumor virus (MMTV) Ags expressed by the tumor (57). Interestingly, a number of studies suggest a possible role of an MMTV-like virus in the etiology of a large proportion of human breast cancers (58, 59). Although direct epidemiological data linking marijuana exposure to increased incidence of breast cancers is not currently available, it is intriguing to speculate that immunocompromised individuals may become increasingly susceptible to MMTV-like infection and to the subsequent development of breast cancers. Therefore, the possibility exists that exposure to marijuana, either through recreational or medicinal use, may lead to increased incidence of immunogenic tumors.

Disclosures

The authors have no financial conflict of interest.

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Prenatal tobacco and marijuana use among adolescents: effects on offspring gestational age, growth, and morphology.

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OBJECTIVE. This longitudinal study examined the effects of tobacco and marijuana use during pregnancy on the gestational age, growth, and morphology of 310 offspring of adolescents. Data were collected during 199 through 1993. METHODOLOGY. The adolescents were drawn from a prena clinic in Pittsburgh, PA. They were interviewed at mid-pregnancy and at delivery to obtain information on tobacco, marijuana, and other substance us before and during pregnancy. Infants were examined 24 to 36 hours after birt RESULTS. The average maternal age was 16.1 (range 12 to 18 years); 70% were African-American. Prenatal tobacco use was associated with reduced birth weight, length, head and chest circumferences, and ponderal index, but not gestational age or the number of morphological abnormalities. Prenatal marijuana exposure was associated with reduced gestational age. Among whites, first trimester marijuana exposure was associated with an increased r of minor physical anomalies. Prenatal marijuana exposure was not associated with any growth outcomes. CONCLUSIONS. These effects of prenatal tobac and marijuana use were prominent despite lower levels of prenatal exposure the offspring of adolescent mothers as compared with the offspring of adult mothers from the same clinic. Young maternal age may increase the offsprin risk of negative effects from prenatal tobacco and marijuana exposure.

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Pharmacology

Prenatal exposure to a cannabinoid agonist produces memory deficits linked to dysfunction in hippocampal long-term potentiation and glutamate release

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Abstract

To investigate the possible long-term consequences of gestational exposure to cannabinoids on cognitive functions, pregnant rats were administered with

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the CB1 receptor agonist WIN 55,212-2 (WIN), at a dose (0.5 mg/kg) that causes neither malformations nor overt signs of toxicity. Prenatal WIN exposure induced a disruption of memory retention in 40- and 80-day-old offspring subjected to a passive avoidance task. A hyperactive behavior at the ages of 12 and 40 days was also found. The memory impairment caused by the gestational exposure to WIN was correlated with alterations of hippocampal long-term potentiation (LTP) and glutamate release. LTP induced in CA3-CA1 synapses decayed faster in brain slices of rats born from WIN-treated dams, whereas posttetanic and short-term potentiation were similar to the control group. In line with LTP shortening, *in vivo* microdialysis showed a significant decrease in basal and K^+-evoked extracellular glutamate levels in the hippocampus of juvenile and adult rats born from WIN-treated dams. A similar reduction in glutamate outflow was also observed in primary cell cultures of hippocampus obtained from pups born from mothers exposed to WIN. The decrease in hippocampal glutamate outflow appears to be the cause of LTP disruption, which in turn might underlie, at least in part, the long-lasting impairment of cognitive functions caused by the gestational exposure to this cannabinoid agonist. These findings could provide an explanation of cognitive alterations observed in children born from women who use marijuana during pregnancy.

Introduction

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Even though marijuana is the most widely used illegal drug among women at reproductive age, reports dealing with the effects of prenatal exposure to this substance of abuse on the length of gestation, fetal growth, and offspring behavior are still controversial (1-4). Confounding factors, such as possible impurities in the drug and concomitant tobacco smoking, may be responsible for inconsistencies in the results reported in studies to date (4, 5). It is likely that many of these conflicting results are due to methodological problems such as the measurement of neonatal outcomes and the context in which the research is conducted. More complex and less understood is the scenario concerning the possible long-term consequences of *in utero* exposure to cannabis derivatives on cognitive functions. In fact, data on this issue are sparse, and the identification of alterations in brain development and adult expression of cognitive and behavioral functions is far from definitive. These inconclusive results may depend on ethical, practical, and interpretative difficulties surrounding research with human subjects (4). In this regard, animal models provide a useful tool for examining the possible developmental and long-term effects of prenatal exposure to cannabinoids (CBs).

Studies performed in adult rats have demonstrated the involvement of a specific CB receptor (CB1) highly expressed in many brain regions (6) in the reinforcing effects of CBs (7) and also in the disruptive effects of either Δ^9-tetrahydrocannabinol (Δ^9-THC) or the synthetic agonist WIN 55,212-2 (WIN) (8) on cognitive processes (9).

In particular, it has been reported that deficits of cognitive functions induced by marijuana use during adulthood could be mainly attributable to the activation of CB1 receptors located in the hippocampus (6, 10, 11), a brain region crucial for certain forms of learning and memory. In this regard, it has been

shown that CBs decrease excitatory postsynaptic currents and disrupt hippocampal long-term potentiation (LTP) (12-14), which is considered the cellular and molecular model for learning and memory (15, 16).

Accordingly, the CB1 receptor-mediated LTP disruption seems to be associated with an inhibition of hippocampal glutamatergic transmission (14, 17), a finding that could be relevant in elucidating the possible electrophysiological and neurochemical mechanisms underlying the effects of CBs on cognitive functions (11, 18).

The aim of the present study was to determine the effect of long-term prenatal exposure to WIN on cognitive function, hippocampal LTP, and hippocampal glutamate release in juvenile and adult rats. Cognitive function, evaluated with a passive avoidance task, was tested 40 and 80 days after birth. LTP was studied in hippocampal slices obtained from 40-day-old rats. Glutamate release was measured by microdialysis in 40- and 80-day-old rats as well as in hippocampal primary cell cultures obtained from pups born from dams exposed to WIN.

Furthermore, because previous clinical findings have reported abnormal motor activity in children of mothers who used marijuana during pregnancy (3, 19), the effect of prenatal WIN exposure on spontaneous motility was analyzed in infant (12-day-old), juvenile (40-day-old), and adult (80-day-old) offspring.

Materials and Methods

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Animal Care.

Experiments were performed in accordance with the guidelines issued by the Italian Ministry of Health (Decreto Legislativo 116/92) and (Decreto Legislativo 111/94-B), the Declaration of Helsinki, and the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the National Institutes of Health.

Animals and Exposure Conditions.

Primiparous Wistar female rats (Harlan SRC, Milan) weighing 250-280 g were housed for 1 week before exposure to males at constant room temperature (20 ± 1°C) and humidity (60%) with lights on 12 h/day (0800 to 2000 h) and food and water available ad libitum. Pairs of females were then placed with single male rats in the late afternoon. Vaginal smears were taken the following morning at 0900 h. The day on which sperm were present was designated as the gestation day 0 (GD 0).

Pregnant rats were treated daily with WIN (0.5 mg/kg) from GD 5 to GD 20. This dose was chosen on the basis of our pilot studies, which showed that prolonged prenatal exposure to a higher WIN dose (1.0 mg/kg) significantly affected reproduction parameters such as dam and pup weight gain as well as litter size at birth. The drug was suspended in 0.3% Tween 80/saline and injected s.c. at the volume of 1.0 ml/kg. Control rats were injected with the vehicle.

Litters were reduced to a standard size of six male pups (when possible) within 24 h after birth. Litters from both control and WIN-exposed groups were then assigned to nonexposed mothers whose pups were born on the same day.

One pup per litter from different litters per treatment group was used in each experiment. Pups were weaned at 21 days of age. Each male pup was used only for a single test and tested once.

Reproduction Data.

Body weights of the dams were taken on GD 0 and GD 20. The number of dams giving birth and the length of pregnancy were determined. Litter size at birth and postnatal mortality (the number of male pups that died before weaning) were evaluated. Body weights of male rats (one pup per litter from 12 control litters and 10 WIN-exposed litters) were recorded.

Behavioral Studies. *Motor activity.*

Motor activity was recorded in an Opto-Varimex apparatus linked to an IBM PC (Columbus Instruments, Columbus, OH) according to the method described by Wedzony *et al.* (20). The apparatus consisted of a cage (42 × 42 × 30 cm) equipped with 15 infrared emitters (spaced at 2.65-cm intervals) located on the *x* and *y* axes 2-3 cm above the floor of the cage (depending on the size of the animal) and an equivalent number of receivers located on the opposite walls. A further line of emitter/receiver pairs was located ≈5 cm (depending on the size of the animal) above the floor of the cage to detect vertical movements (i.e., rearings). Each interruption of a beam generates an electric impulse scored by a digital counter.

Procedure.

The amount of time spent in ambulatory activity was analyzed by using AUTO-TRACK software (Columbus Instruments, Columbus, OH). Ambulatory activity was defined as a trespass of three consecutive photo-beams, whereas other movements (e.g., repeated interruption of the same photo-beams) were regarded as stereotypic movements. Resting time was calculated as the amount of time during which there were neither ambulatory nor stereotypic movements. Furthermore, vertical activity was measured by recording the number of horizontal beams that were broken by the rearings of the animal.

Tests (5-min sessions) were carried out in a 1 × 1 × 2 m sound-attenuating cabin (Amplifon G-type cabin) illuminated by a 20-W white light source suspended 2 m above the apparatus. Background noise of 42 dB sound pressure level was produced by a fan. Different groups of animals were tested at 12, 40, and 80 days of age. Experimental groups: (*i*) vehicle-treated groups (10 animals) and (*ii*) WIN-treated groups (8 animals). Tests were carried out between 0900 and 1400 h.

Passive avoidance behavior.

A "step-down" apparatus was used according to the method extensively described by Trabace *et al.* (21). It consisted of a compartment (25 × 24 × 24 cm) constructed of black Plexiglas and equipped with a grid

floor to which an elevated compartment (13 × 24 × 16 cm) with a solid Plexiglas floor was attached. A guillotine door (9 × 10 cm) separated the opening between the elevated compartment and the large compartment. A 25-W lamp illuminated the elevated compartment while the large compartment remained dark. Scrambled foot shocks were delivered from a Letica shock generator (LI 2750 Unit, Barcelona). The experiments were performed in a sound-attenuating chamber (Amplifon G-type cabin) that was dark except for illumination of the elevated compartment of the apparatus.

Procedure.

Each animal was removed from the home cage and placed in a holding cage adjacent to the apparatus. Two minutes later, the rat was placed in the illuminated compartment, and, after a 10-s delay, the guillotine door was raised. The time taken by the animal to completely enter into the dark compartment was measured (approach latency) and taken as an index of emotional, nonassociative behavior.

A single 2-s inescapable scrambled foot shock (0.8 mA) was delivered immediately after the rat entered the dark compartment. Twenty-four hours after this session (acquisition trial), each animal was tested for memory retention. The animal was placed in the elevated compartment and latency to re-enter (avoidance latency) the dark compartment was recorded and assumed to be a measure of memory retention. Both acquisition and retention trials lasted for a maximum observation time of 180 s. The experiments were conducted in 40- and 80-day-old male offspring of either control or WIN-exposed mothers, each group consisting of eight rats.

Electrophysiological Studies.

The electrophysiological experiments (see ref. 22 for details) were performed in 40-day-old offspring of either vehicle-exposed ($n = 10$) or WIN-exposed ($n = 9$) mothers. Transverse hippocampal slices were prepared following standard methods. Briefly, rats were decapitated under deep anesthesia by halothane (4.0% in O_2), and the brain was rapidly removed. Slices (350 μm thick) were cut in chilled Ringer solution with a vibroslicer (VSL, WPI, Sarasota, FL), incubated at room temperature (20 ± 2°C) for at least 60 min, and then individually transferred to the recording (submerged) chamber. At least two slices from each animal were tested. Ringer medium contained 124 mM NaCl, 3.5 mM KCl, 1.25 mM NaH_2PO_4, 22.0 mM $NaHCO_3$, 10.0 mM dextrose, 1.0 mM $MgCl_2$, 2.0 mM $CaCl_2$. The solution was maintained at pH 7.4 by continuous bubbling with 5% CO_2 in O_2.

The procedure was as follows. Field-excitatory postsynaptic potentials (f-EPSPs) were recorded from stratum radiatum of CA1 pyramidal cells in response to monopolar stimuli (20 μs-duration) delivered to the Schaffer collateral/commissural pathway via platinum electrodes. Recording electrodes were filled with the medium (1-2 MΩ). Synaptic responses were sampled at 5-10 kHz. Acquisition and analysis were performed by a pCLAMP 5.5/Digidata 1200 system (Axon Instruments Inc., Foster City, CA). The evoked f-EPSPs were measured as the slope of their rising phase after the presynaptic volley. An *I/O* curve was constructed for each slice by plotting increasing single stimulus intensity (scan: 50 to 1,000 μA) vs. the evoked f-EPSP. The current intensity required to produce 50% of maximal response (EC_{50}) was used to assess the synaptic excitability and was used for test stimulation and tetanization.

Samples of f-EPSP were taken every 5 min, averaging 10 consecutive responses (22). Tetanization consisted of two trains of stimuli (100 Hz for 1.0 s at 25-s intervals) delivered after at least 30 min of baseline. Responses were followed up to 180 min and were considered potentiated if their slope was ≥20% of baseline.

The three temporal phases of f-EPSP changes, i.e., posttetanic potentiation (PTP), short-term potentiation (STP), and LTP expression (or maintenance) were distinguished as indicated (15, 16, 22, 23).

Neurochemical Studies. *Microdialysis.*

In vivo experiments were performed in the offspring of WIN-treated and vehicle-treated dams, at the age of 40 and 80 days. Under halothane anesthesia (1.5% mixture of halothane/air), animals were mounted in a David Kopf stereotaxic apparatus, and a microdialysis probe (1 mm dialyzing membrane length) was implanted into the hippocampus. The coordinates relative to bregma were as follows: anteroposterior, −5.2; mediolateral, ±4.0; and dorsoventral, −3.8 mm (24). After the implantation, the probe was secured to the skull with methacrylic cement. Microdialysis measures were performed after at least 36 h of recovery.

Procedure.

On the day of the experiment, the probe was perfused with an artificial cerebrospinal fluid (148 mM NaCl/2.7 mM KCl/1.2 mM $CaCl_2$/0.85 mM $MgCl_2$/2.7 mM glucose) at a constant flow rate (2 μl/min) via a microinfusion pump. At least 300 min later, dialysates were collected every 20 min, and glutamate content was measured by HPLC. The average concentration of three successive stable samples (variation ≤10%) was considered as baseline glutamate outflow. Thereafter, the probe was perfused (10 min) with an isotonic artificial cerebrospinal fluid containing 50 mM KCl. This medium was then replaced with the original one, and further four samples were collected.

Histology.

At the end of each experiment, the probe location was verified in 30-μm-thick coronal cryostat sections. Only those animals in which the probe was correctly located were included in the study.

Hippocampal cell cultures.

Hippocampal cells were prepared from 1-day-old rats (25) born from mothers that had received the WIN-vehicle (control) or WIN during pregnancy. Briefly, neurons were plated on poly-L-lysine (5 μg/ml)-coated dishes at a density of 2.5×10^6 cells per dish and cultured in Eagle's Basal Medium supplemented with inactivated FCS, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamycine. Cultures were grown at 37°C in a humidified atmosphere, 5% CO_2/95% air. Cytosine arabinoside (10 μM) was added within 24 h of plating to prevent glial cell replication. The cultures were used in experiments after 8 days *in vitro*.

Procedure.

On the day of the experiment, the cells were rinsed twice by replacing the culture medium with Krebs-Ringer bicarbonate buffer (37°C). Thereafter, five consecutive fractions were collected renewing this solution (400 μl) every 30 min. The first three samples were used to assess basal glutamate levels while, to evoke endogenous glutamate, cells were treated with an isotonic Krebs solution containing 20 mM KCl, applied 20 min before the end of the fourth fraction.

Endogenous glutamate assay.

Endogenous glutamate was quantified by using an HPLC/fluorimetric detection system, including precolumn derivatization *o*-phtaldialdeihyde reagent and a Chromsep 5 (C18) column. The mobile phase consisted of 0.1 M sodium acetate, 10% methanol, and 2.5% tetrahydrofurane, pH 6.5 (0.75 ml/min; ref. 26).

Statistical Analysis.

The reproduction data were analyzed by overall one-way or two-way ANOVAs followed by post hoc tests (Tukey's test) for individual comparisons between groups. Fisher's exact test was used where appropriate.

The analysis of motor activity data were based on overall two-way ANOVAs followed by post hoc tests (Tukey's test).

Mann-Whitney *U* test was used to analyze the passive avoidance data. The electrophysiological results were evaluated by a two-way ANOVA for repeated measures followed by Tukey's test or Student's *t* test, where appropriate. Data obtained from neurochemical studies were analyzed by Student's *t* test for grouped data.

Substances.

WIN mesylate {(R)-(+)-[2,3-dihydro-5-methyl-3-(4-morpholinyl-methyl)pyrrolo (1,2,3-de)-1,4-benzoxazin-6-yl]-1-naphthalenylmethanone} was obtained from Tocris Cookson (Bristol, U.K.). The culture dishes were purchased from Nunc. FCS and basal Eagle's medium were obtained from GIBCO. Poly-L-lysine, trypsin, soybean trypsin inhibitor, DNase, cytosine arabinoside, gentamycine sulfate, and glutamine were obtained from Sigma.

Results

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General Reproduction Data.

General reproduction data are reported in Table 1. Overall one-way ANOVAs showed that prenatal treatment with WIN did not significantly affect dam weight gain [$F = 3.65$, $df = 1/20$, not significant (n.s.)], pregnancy length ($F = 0.33$, $df = 1/20$, n.s.), and litter size at birth ($F = 1.71$, $df = 1/20$, n.s.). Moreover, an overall two-way ANOVA for repeated measures showed that prenatal exposure to

the CB1 receptor agonist did not influence male pup weight gain: ($F_{treatments} = 0.01$, $df = 1/20$, n.s.; $F_{ages} = 1638$, $df = 2/4^{\prime}$, $P < 0.001$; $F_{treatments \times ages} = 0.52$, $df = 2/40$, n.s.). Finally, Fisher's exact test revealed that WIN treatment caused neither hypothermia, catatonia, or hypomotility in dams, nor postnatal toxicity or teratogenesis in male pups (data not shown).

Table 1. Reproduction data

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Behavioral Studies. *Motor activity.*

An overall two-way ANOVA for repeated measures of the ambulatory time (Fig. 1) showed the following effects: $F_{treatments} = 12.72$, $df = 1/16$, $P < 0.005$; $F_{ages} = 100.18$, $df = 2/32$, $P < 0.001$; $F_{treatments \times ages} = 7.80$, $df = 2/32$, $P < 0.001$. Individual comparisons (Tukey's test) revealed that prenatal treatment with WIN significantly increased the ambulatory time of the offspring at both postnatal day (PND) 12 ($P < 0.05$) and 40 ($P < 0.01$). No significant differences were observed in ambulatory activity between the two groups at 80 days of age.

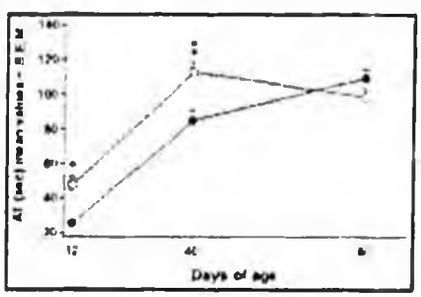

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Fig. 1. Effects of prenatal treatment with WIN on motor activity in 12-, 40-, and 80-day-old rats (●, vehicle; ○, WIN). Each point represents the mean ± SEM of the ambulatory time (AT) spent by rats in 5-min trials. *n* was 10 and 8 for vehicle- and WIN-exposed rats, respectively. *, $P < 0.05$; **, $P < 0.01$ (vs. controls; Tukey's multiple comparison test).

Furthermore, overall two-way ANOVAs for repeated measures of both stereotypic time and rearings did not reveal any significant change between controls and WIN-exposed offspring at all ages (PND 12, 40, and 80) considered in the present study (data not shown).

Passive avoidance behavior.

As shown in Fig. 2, during the first (acquisition) trial 40- and 80-day-old rats from the control group showed approach latencies that did not differ significantly with respect to animals prenatally exposed to WIN. However, when the trial was repeated 24 h later (retention trial), the avoidance latencies of the WIN-exposed group were significantly shorter than those of control animals ($P < 0.01$, Mann-Whitney

U test).

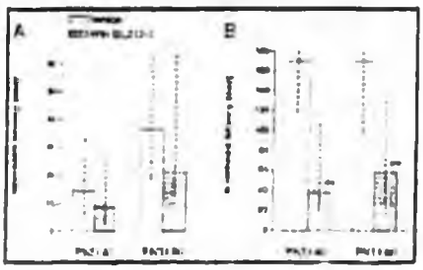

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Fig. 2. Effect of prenatal WIN exposure on approach latency (*A*) and avoidance latency (*B*) measured 24 h later (retention) of 40- and 80-day-old offspring in a passive avoidance task. Data represent median values and interquartiles (dashed line). **, $P < 0.01$ with respect to relative control (Mann-Whitney *U* test).

Electrophysiological Studies. *Synaptic excitability.*

Changes in basal synaptic excitability in hippocampal slices from 40-day-old rats were investigated, comparing the current intensity required to produce 50% of maximal response (excitatory current 50) before tetanization in both groups and by the evaluation of the number of slices exhibiting PTP.

Although the EC_{50} in slices from WIN-treated rats (WIN-slices) was found to be slightly higher than it was in controls, no statistical significance was reached (Student's *t* test). This result indicates that the responsiveness of CA3-CA1 synapses to electrical stimuli was not affected by the treatment.

Moreover, the first potentiation, which immediately follows tetanization (PTP), was found to be comparable in the two groups [255.90 ± 18.07 and 229.18 ± 23.43% for vehicle- and WIN-treated animals, respectively (Student's *t* test)]. Furthermore, the occurrence of slices showing a PTP of at least 200% was similar [22/22 and 21/21 in slices from control and WIN-treated group, respectively (Table 2)].

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Table 2. Number of slices from 30- to 40-day-oid offspring showing PTP, STP, and LTP of f-EPSP

Taken together, these results indicate that no alterations in basal synaptic excitability were evident in slices from prenatally WIN-exposed rats.

Time course of STP and LTP.

In control slices the decay of f-EPSP potentiation after tetanization followed a typical biphasic curve (Fig. 3*A*). Thus, in agreement with previous studies (15, 16, 23, 27), the f-EPSP slope in control slices showed a first fast decremental phase lasting 15 to 20 min (STP), which then slowly decayed over the

observation time (180 min).

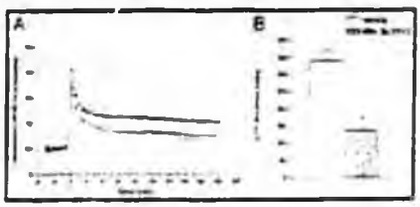

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Fig. 3. Prenatal WIN selectively suppresses LTP maintenance but not its induction. (*A*) Time course of the averages of f-EPSP slopes from slices obtained from 40-day-old offspring of vehicle-treated (●) and WIN-treated dams (○). Values of f-EPSP slopes have been normalized to the pretetanus period. Each point represents the average ± SEM of 10 consecutive responses taken every 5 min. Just after tetanization (given at time −5 min), the values of f-EPSP first potentiation (i.e., the PTP) were 255.90 ± 18.07 and 229.18 ± 23.43% for vehicle- and WIN-treated groups, respectively (not significant, Student's *t* test). (*B*) Duration of LTP. Evoked f-EPSPs were considered potentiated until their slope was ≥20% with respect to baseline. For the vehicle group, LTP duration was estimated by fitting analysis of the curve in *A* to calculate the interception point where this curve asymptotically subsided to a value of +20%. It occurred at 334.45 ± 25.36 min after tetanus. However, the curve describing LTP expression of the WIN-treated group (data from *A*) returned to +20% of baseline in 136.87 ± 12.18 min. Bars represent the mean ± SEM obtained from 22 and 21 slices from vehicle- and WIN-exposed rats, respectively. *, $P < 0.001$ vs. vehicle (Student's *t* test).

in line with the PTP results (see above), the time course of the early phase of f-EPSP de-potentiation (STP) was similar in both treated and control groups (Fig. 3*A*). Indeed, a two-way ANOVA showed no significant deviation between the two curves ($P < 0.09$) in the STP interval (from 5 to 20 min).

Thereafter, however, the averaged curve describing the LTP-expression phase decayed faster in slices from WIN-treated than in vehicle-treated offspring (Fig. 3*A*). Accordingly, the deviation between the two curves, in the 20- to 180-min interval after tetanization, was statistically significant ($P < 0.01$, two-way ANOVA).

Moreover, whereas a typical time course of LTP was seen in 20/22 tested slices from control animals, the potentiation remained above +20% for >60 min in only 3 of 21 slices from the WIN group (Table 2).

As shown by fitting analysis, the interception points at which the averaged curves of LTP, asymptotically subsided to a value of +20%, with respect to the baseline, were at 334.45 ± 25.36 and 136.87 ± 12.18 min, after tetanus, in control and WIN slices, respectively (Fig. 3*B*).

Neurochemical Studies. *Hippocampal cell cultures experiments.*

Basal extracellular glutamate levels were measured in hippocampal cell cultures obtained from 1-day-old pups. As shown in Fig. 4*A*, glutamate levels were found to be significantly lower ($P < 0.01$; Student's *t* test) in animals born from mothers exposed to WIN during pregnancy than in those born from

vehicle-treated mothers.

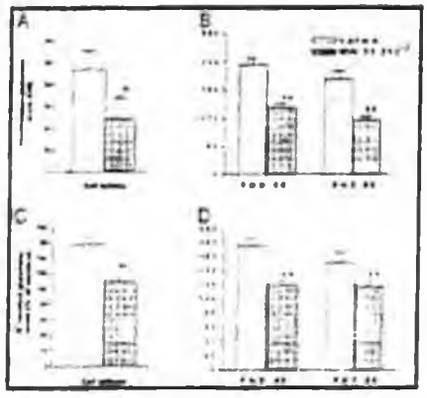

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Fig. 4. Effect of prenatal exposure to WIN on basal (*A* and *B*) and K^+-evoked (*C* and *D*) extracellular hippocampal glutamate levels in cell cultures (*A* and *C*) and *in vivo* at 40 and 80 days of age (*B* and *D*). Each column represents the mean ± SEM obtained from 22 and 11 replications for *in vitro* and *in vivo* experiments, respectively. **, $P < 0.01$ vs. vehicle (Student's *t* test).

Bath application of KCl (20 mM) increased glutamate extracellular levels in both cell cultures. However, the increase was significantly lower ($P < 0.01$; Student's *t* test) in cultures of rats born from WIN-treated mothers than in those obtained from control pups (Fig. 4*C*).

Microdialysis in vivo.

Basal extracellular hippocampal glutamate levels, evaluated as the mean of three stable dialysates, were significantly lower ($P < 0.01$; Student's *t* test) in both 40- and 80-day-old rats born from mothers treated with WIN during pregnancy than in those born from mothers treated with the vehicle (Fig. 4*B*).

A 10-min pulse of high K^+ (50 mM) solution significantly increased glutamate efflux in both groups of animals. However, the K^+-evoked glutamate efflux from the hippocampus of rats born from mothers exposed to WIN during pregnancy, was significantly lower ($P < 0.01$; Student's *t* test) than the enhancement observed in rats born from mothers treated with vehicle during pregnancy (Fig. 4*D*).

Discussion

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The present study, by combining different methodological approaches, provides evidence that maternal exposure to the CB1 receptor agonist WIN induces impairment of memory retention capacities in the offspring. This impairment is associated with alterations of hippocampal LTP and glutamate outflow.

WIN-exposed offspring were also characterized by motor hyperactivity during infantile and juvenile, but not adult, periods.

Memory impairment in prenatally WIN-exposed rats, assessed by the disruption in the retention of a passive avoidance task, seems to be a persistent condition, present at both 40 and 80 days of age.

Memory impairment does not appear to be attributable to alterations of a nonassociative nature, because approach latency, measured during the acquisition trials of the learning task, remained unchanged.

WIN-treated dams did not show hypothermia, catatonia, or hypomotility, which are typically induced by the high and/or moderate exposure to CBs (8). Moreover, the dose of WIN used in the present study (0.5 mg/kg/die s.c.) produced neither gross malformations nor overt signs of toxicity, and it failed to alter reproductive parameters, such as dam and pup weight and weight gain. Furthermore, litters of WIN-treated dams were assigned to untreated dams to avoid confounding factors generated during lactation as well as malnutrition.

However, memory deficit produced by prenatal WIN may be dissociated from the hyperactivity, which has been reported to be caused postnatally by WIN (28), because the latter was present at 40 but not at 80 days of age, whereas the former was present at both periods.

Memory impairment observed in offspring exposed prenatally to WIN was correlated to alterations in both hippocampal LTP, a widely accepted cellular and molecular model for learning and memory (15, 16), and hippocampal glutamate release.

LTP was assessed in brain slices from 40-day-old rats, whereas spontaneous and K^+-evoked glutamate release was measured *in vivo* at 40 and 80 days of age, as well as in cell cultures obtained from 1-day-old pups.

Slices from WIN-treated animals showed a reduced ability to maintain LTP over time, whereas basal synaptic excitability and LTP induction phases (PTP and STP) were normal.

These results are in agreement with previous observations made in brain slices from adult rats showing that bath application of low concentrations of Δ^9-THC selectively reduced LTP duration but not the extent of PTP (12).

However, other authors have reported that CB1 receptor activation by WIN, in slices from adult rodent brain, suppressed both early (induction) and late (maintenance/expression) phases of LTP in hippocampal CA3-CA1 synapses (11, 14, 29). The inhibitory effect of CBs on hippocampal LTP has been attributed to the reduction in presynaptic glutamate release and the consequent suppression of *N*-methyl-D-aspartate-mediated entry of postsynaptic Ca^{2+}, necessary for LTP induction, rather than to a direct modulation of postsynaptic ionic channels (11, 14, 30).

According to this hypothesis, the microdialysis data have shown that basal and K^+-stimulated extracellular hippocampal glutamate release was significantly lower in animals born from WIN-treated dams than in control animals.

Therefore, it might be suggested that in rats exposed prenatally to WIN, glutamate release is sufficiently preserved to activate *N*-methyl-D-aspartate receptors responsible for LTP induction, but it is not

sufficiently sustained to stimulate postsynaptic metabotropic and α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors involved in LTP maintenance (15).

The reduced glutamate outflow seems to be a precocious and persisting consequence of prenatal exposure to the CB agonist: it is already present in cell cultures, obtained from 1-day-old WIN-exposed pups, and persists unmitigated at 40 and 80 days of age.

Although the reduction of glutamate outflow could explain the disruption of LTP induced by gestational exposure to the CB1 receptor agonist, the electrophysiological alterations may, in turn, represent a neuronal substrate responsible for the selective retention deficit (reduction of avoidance latencies in a passive avoidance task) that was observed in the offspring of mothers treated with WIN during pregnancy.

Thus, it may be hypothesized that gestational exposure to the CB produces an irreversible alteration to endogenous CB1 systems in the developing brain (29, 31), possibly leading to a long-term disruption of hippocampal function. Accordingly, CB1 receptors are already measurable at GD 14 in a variety of brain structures including hippocampus (32).

Additional studies are needed to clarify whether the effects caused by WIN are reproduced by Δ^9-THC and whether they may be prevented by CB1 receptor antagonists such as SR 141716.

Concerning the clinical relevance of the present study, it is important to estimate, by extrapolation, whether the dose of WIN administered compares with that of Δ^9-THC absorbed by cannabis users.

Previous studies have estimated that 5 mg/kg Δ^9-THC in rats corresponds to a moderate exposure of the drug in humans, correcting for the differences in route of administration and body weight surface area (33, 34, 35).

However, WIN has been found to be 3-10 times more potent than Δ^9-THC, depending on the administration route and the endpoints considered (8, 36, 37). This estimate is consistent with the relative K_i of each compound for CB1 receptors in brain membranes, i.e., 2-12 nM vs. 35-80 nM for WIN and Δ^9-THC, respectively (38). Based on these considerations, the dose of WIN used in the present study might correspond to a moderate, or even to a low, exposure to cannabis in humans.

The present results are in line with clinical data showing that the consumption of marijuana by women during pregnancy has negative consequences on the cognitive functions of their children. In particular, memory has been reported to be negatively associated with daily marijuana use, and this statistical association remained after checking for confounding variables (39).

Moreover, the increased motor activity observed in both infant and juvenile offspring of WIN-treated dams is consistent with data showing that children prenatally exposed to marijuana were rated, at a prepuberty age, as hyperactive, inattentive, and particularly impulsive (3, 19).

Whatever the mechanism of action of prenatal exposure to WIN, our results suggest that alterations of

hippocampal glutamategic function may underlie, at least in part, the subtle impairment of cognitive processes induced by gestational marijuana exposure (1, 4, 39).

Acknowledgements

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Abbreviations

CB, cannabinoid; f-EPSP, field excitatory postsynaptic potential; LTP, long-term potentiation; PTP, posttetanic potentiation; STP, short-term potentiation; GD, gestation day; PND, postnatal day; WIN, WIN 55,212-2; Δ^9-THC, Δ^9-tetrahydrocannabinol.

Footnotes

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Marijuana Use and Birth Defects

When parents abuse alcohol or drugs, the consequences can be devastating for their children. Children of alcoholic or drug-dependent parents can suffer neglect, developmental hindrances, and emotional and physical abuse. However, people who abuse substances can also damage the health of their unborn children. For example, a woman who smokes marijuana during pregnancy can put the fetus at risk for a host of dangerous problems, such as low birthweight, developmental difficulties, and even drug addiction. Similarly, a woman who drinks alcohol while pregnant—perhaps before she even realizes she's pregnant—can cause heart defects, growth retardation, and serious neurological injury to the fetus (commonly known as fetal alcohol syndrome, or FAS).

Much research has shown that alcohol and drug use on the part of men and women can impair fertility and/or lead to birth defects in their children. Despite these known risks, a National Institute on Drug Abuse study on alcohol and other drug use among pregnant women found that 5.5 percent of the study participants used illicit drugs while pregnant, 18.8 percent used alcohol, and 20.4 percent used tobacco.[1] These figures are even higher for teen mothers. In another study, one-third of mothers between the ages of 12 and 18 used marijuana before their pregnancies, and of those, over half used it at some time during their pregnancies.[2] With statistics like these, it's important that teens understand the additional risks of alcohol and drug use, particularly if they are sexually active.

Specifically, how does marijuana affect the fetus? Like alcohol and tobacco, marijuana use has been linked to low birth weight and premature babies. One study showed that marijuana use by the mother is associated with slow embryo growth and spontaneous abortion in the early stages of pregnancy. Other studies have shown that marijuana causes FAS-like symptoms in newborns, such as abnormally shaped heads, small size, and nervous-system difficulties. Research also suggests that the fetuses of teen mothers, as opposed to those of adult mothers, may be especially vulnerable to the damage marijuana causes, especially during the first trimester (when the teen may not even know she's pregnant).[2] Symptoms such as excessive trembling and withdrawal-like irritability in newborns have also been associated with heavy marijuana use by the mother.

In addition, THC, the active component in marijuana, can be passed from mother to infant through breastfeeding.[1] This chemical is more concentrated in the mother's breastmilk than it is in her blood, and use of marijuana by breastfeeding mothers has been linked to motor development problems in newborns.

In general, it is important to remember that the fetus can be exposed to anything that enters the mother's body. THC can pass freely through the placenta. If marijuana poses a health risk to the mother's body, it also poses a potential risk to the fetus she carries in her body.

For males, studies have established that marijuana can lower sperm count, but the drug can also cause slow-moving or abnormally-shaped sperm, which can ultimately lead to miscarriages, low fetal birth weight, or other health problems. [3] Also, simply smoking marijuana around a pregnant woman could endanger both the woman and the fetus if she inhales the secondhand smoke.

Does your teen want to be a parent someday? She may feel that she's too young to worry about children right now, but if she becomes addicted to drugs or alcohol, her addiction could last well into her adult years. Studies indicate that female drug users usually reduce their drug intake after they discover they are pregnant, but they often do not or cannot quit completely.[4] This is a testament to just how addictive drugs can be, even at a time when it is especially important to stay healthy. When your teen finally decides she does want to be a parent, she may have a very difficult time quitting.

Make sure your teen knows that substance abuse can permanently damage the life chances of his future children. The National Council on Alcoholism and Drug Dependence established National Alcohol- and Other Drug-Related Birth Defects Awareness Week (May 13-19) to recognize and help educate others about the dangers of prenatal exposure to alcohol and drugs. This is one kind of birth defect that is highly preventable, so take this opportunity to talk to your teen about these and other serious risks of substance abuse.

For more information, see the following sites:

The National Council on Alcoholism and Drug Dependence, "Alcohol- and Other Drug-Related Birth Defects."

Marijuana Interferes With Early Pregnancy

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In utero marijuana exposure associated with abnormal amygdal dopamine D2 gene expression in the human fetus.

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Department of Clinical Neuroscience, Psychiatry Section, Karolinska Institut 171-76 Stockholm, Sweden.

BACKGROUND: Marijuana (Cannabis sativa) is the illicit drug most used b pregnant women, and behavioral and cognitive impairments have been documented in cannabis-exposed offspring. Despite the extensive use of marijuana, very limited information exists as to the consequences of prenatal cannabis exposure on the developing human brain. METHODS: We optimiz an in situ hybridization histochemistry technique to visualize mRNA expression in midgestation (weeks 18-22) human fetal specimens from moth with and without documented evidence of cannabis use during pregnancy. Tl cannabinoid receptor type 1 (CB(1)) and major dopamine receptor subtypes, (1) and D(2), were examined in the striatum and mesocorticolimbic structure (amygdala and hippocampus). RESULTS: Adjusting for various covariates, found a specific reduction, particularly in male fetuses, of the D(2) mRNA expression levels in the amygdala basal nucleus in association with maternal marijuana use. The reduction was positively correlated with the amount of maternal marijuana intake during pregnancy. No significant cannabis-related alterations were detected in the hippocampus or caudal striatum for the D(2), (1), and CB(1) mRNA levels, although alcohol showed significant contributi to striatal D(1)/D(2) expression. CONCLUSIONS: These human fetal findin suggest that in utero cannabis exposure may impair distinct mesocorticolimb neural systems that regulate emotional behavior.

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Marijuana impairs growth in mid-gestation fetuses.

Hurd YL, Wang X, Anderson V, Beck O, Minkoff H, Dow-Edwards D.

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Karolinska University Hospital, Stockholm, Sweden.

Marijuana (Cannabis sativa) is the most commonly used illicit drug by pregnant women, but information is limited about the effects of prenatal cannabis exposure on fetal development. The present study evaluated the influence of early maternal marijuana use on fetal growth. Women electing voluntary saline-induced abortions were recruited at a mid-gestational stage (pregnancy (weeks 17-22), and detailed drug use and medical histories were obtained. Toxicological assays (maternal urine and fetal meconium) were us(in conjunction with the maternal report to assign groups. Subjects with documented cocaine and opiate use were excluded. Main developmental outcome variables were fetal weight, foot length, body length, and head circumference; ponderal index was also examined. Analyses were adjusted f(maternal alcohol and cigarette use. Marijuana (n=44)- and nonmarijuana (n=95)-exposed fetuses had similar rates of growth with increased age. However, there was a 0.08-cm (95% CI -0.15 to -0.01) and 14.53-g (95% CI 28.21 to 0.86) significant reduction of foot length and body weight, respectively, for marijuana-exposed fetuses. Moreover, fetal foot length development was negatively correlated with the amount and frequency of marijuana use reported by the mothers. These findings provide evidence of a negative impact of prenatal marijuana exposure on the mid-gestational fetal growth even when adjusting for maternal use of other substances well knowr to impair fetal development.

PMID: 15734273 [PubMed - in process]

[redacted]

Drug Abuse Warning Network

The DAWN Report

AUGUST 2003

Marijuana-related Emergency Department Visits by Youth

In Brief

- According to the Drug Abuse Warning Network (DAWN), marijuana is the most frequently reported drug in emergency department (ED) visits related to drug abuse in youth age 12 to 19.
- In 2001, youth age 12 to 19 made an estimated 26,706 ED visits related to the abuse of marijuana or marijuana with other substances.[1] More than 60 percent (16,516) of these visits involved youth age 12 to 17.
- From 1994 to 2001, youth age 18 to 19 had the highest rates of marijuana-related ED visits per 100,000 population; adults age 35 and over had the lowest rates. Rates for youth age 12 to 17 fell between these two extremes.
- The rate of marijuana-related ED visits[2] among youth has been increasing. For youth age 12 to 17, the rate of marijuana-related ED visits rose 126 percent from 1994 to 2001, while their overall rate of drug-related ED visits was stable. For youth age 18 to 19, the rate of marijuana-related visits increased 149 percent over this time period.
- More than half of marijuana-related ED visits among youth age 12 to 17 involve other drugs,[3] particularly alcohol, cocaine, and amphetamines.
- When marijuana alone was implicated in the ED visit, *psychic effects* was the most commonly cited motive for using the drug (in 60% of cases for youth age 12 to 17), and *unexpected reaction* was the most commonly cited reason for the ED visit (40% of cases).

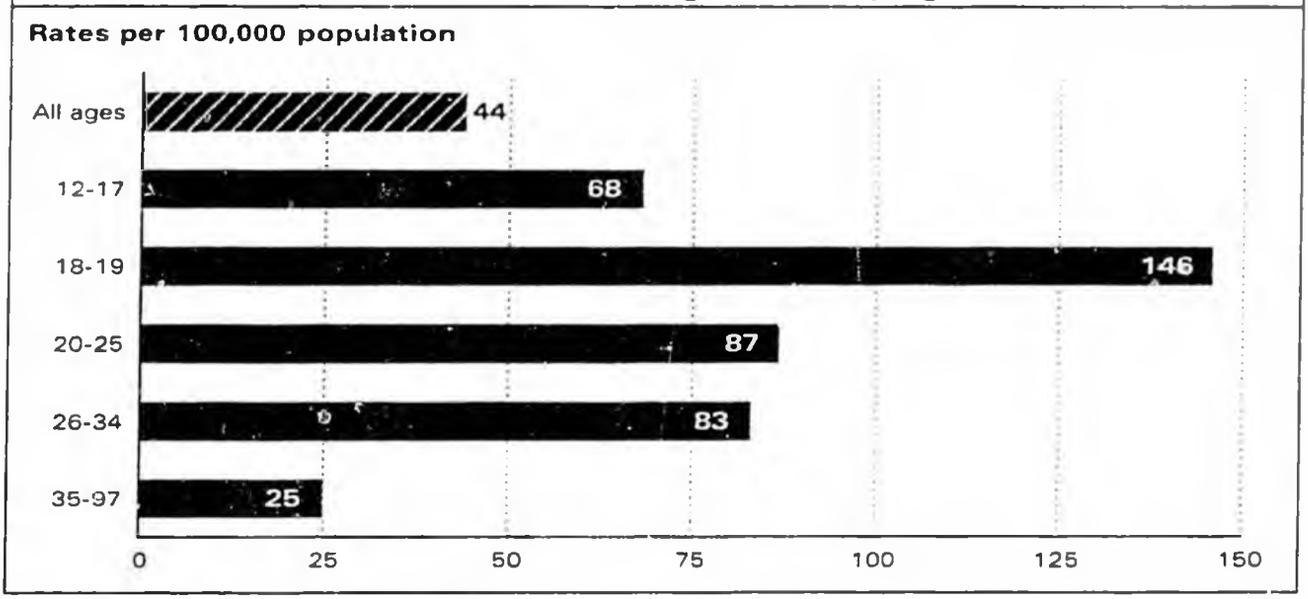
[1] The number of DAWN visits does not represent individuals because a patient may make multiple visits to an ED.

[2] Rates are expressed as the number of ED visits per 100,000 population in the age group.

[3] Up to 4 drugs plus alcohol may be reported for a single visit.

FIGURE 1

Marijuana in ED visits related to drug abuse, by age: 2001



Marijuana is more frequently reported than other drugs in ED visits among youth.

Marijuana abuse by youth is a major and growing problem in the U.S. An estimated 3.6 million youth age 12 to 17 reported using marijuana in 2001,[4] an increase of 14 percent since 2000.

Patients who present to hospital EDs for problems related to marijuana abuse represent only a small fraction of users. Still, in ED visits related to drug abuse, DAWN finds that marijuana is reported more frequently than any other drug for youth age 12 to 19.

In 2001, youth age 12 to 17 made an estimated 16,516 ED visits related to the abuse of marijuana or marijuana with other substance(s).[5] Youth age 18 to 19 added another 10,190 ED visits involving marijuana.

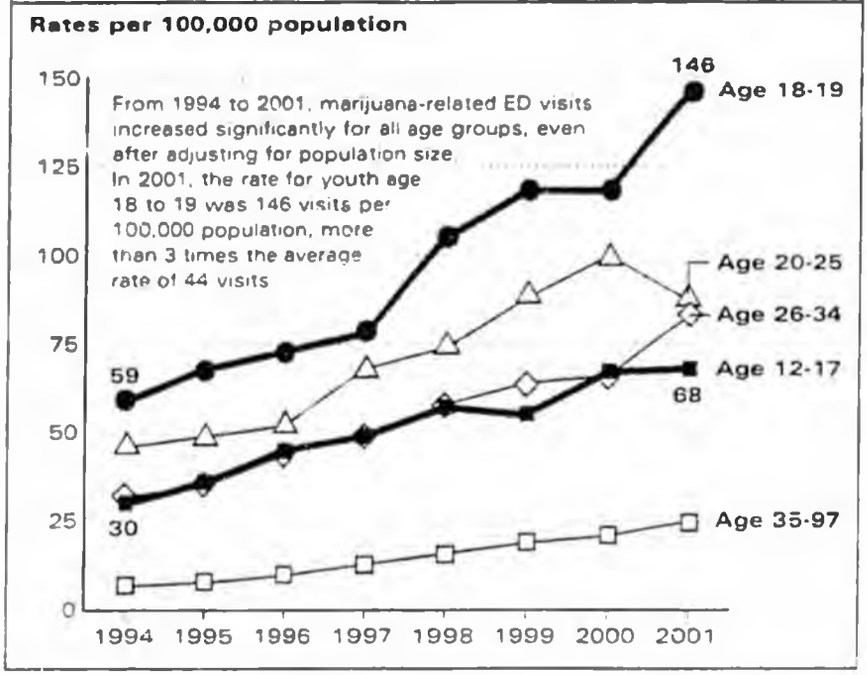
Overall, marijuana was a factor in more than 1 in 4 drug-related ED visits among youth (27% of 61,695 visits for age 12 to 17, and 29% of 34,578 visits for age 18 to 19).

Youth account for a disproportionate number of marijuana-related ED visits.

In 2001, youth age 12 to 17 made up 15 percent of marijuana-related ED visits and 28 percent of marijuana-only visits. By contrast, they were 10 percent of the population and 10 percent of all DAWN ED visits.

FIGURE 2

Marijuana-related ED visits, by age group: 1994 to 2001



Youth age 18 to 19 accounted for 9 percent of marijuana-related visits and 14 percent of marijuana-only visits. This age group was only 3 percent of the population and 5 percent of DAWN ED visits overall.

Marijuana-related ED visits are highest for youth age 18 to 19.

In 2001, there were 68 marijuana-related visits per 100,000 youth age 12 to 17. For youth age 18 to 19, the rate was 146 visits per 100,000, the highest of all age groups (**Figure 1**). By contrast, the rate across all age groups was 44 visits per 100,000.

Since 1994, youth age 18 to 19 have had the highest rates of marijuana-related ED visits, and adults age 35 and over have had the lowest. Rates for youth age 12 to 17 fell between these two extremes (**Figure 2**).

Marijuana-related ED visits are increasing faster than drug-related visits overall.

Marijuana-related ED visits have been increasing much faster than drug-related visits overall, with increases evident in every age group. The rate of marijuana-related visits for all ages increased 151 percent, from 17 to 44 per 100,000 population, from 1994 to 2001. By contrast, the rate of drug abuse-related ED visits overall increased a mere 12 percent, with many age groups showing no increase at all.

Among youth age 12 to 17, the rate of marijuana-related visits increased 126 percent from 1994 to 2001, while the rate of drug abuse visits overall did not increase at all.

Among youth age 18 to 19, the rate of marijuana-related visits increased 149 percent from 1994 to 2001, while the rate of drug abuse visits overall increased only 20 percent.

[4] Office of Applied Studies, Substance Abuse and Mental Health Services Administration (8/2002) *Results From the 2001 National Household Survey on Drug Abuse (NHSDA): Volume III Detailed Tables.* Rockville, MD.

[5] Up to 4 drugs plus alcohol may be reported for a single visit.

Marijuana is often reported with other drugs.

Why do individuals go to EDs as a result of marijuana abuse? In many cases, marijuana is present along with other drugs.

In 2001, almost half (46%) of marijuana-related visits among youth age 12 to 17 involved marijuana as the only drug reported. In 1994, this number was 35 percent[6] (**Figure 3**).

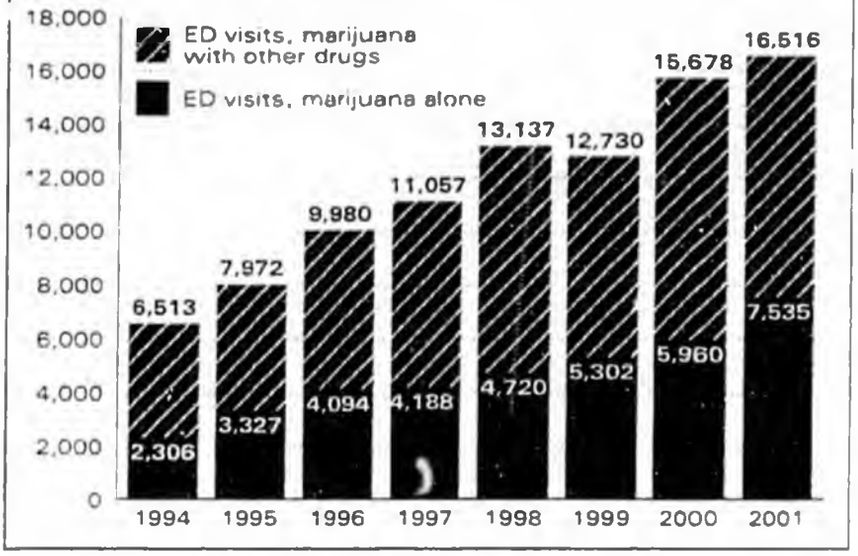
Despite this growth in marijuana-only visits, over half of the marijuana-related ED visits continue to involve more than one drug.

In 2001, alcohol was reported most often with marijuana for all age groups. Among youth age 12 to 17, alcohol was present in more than a quarter (26%) of marijuana-related ED visits. In this group, 14 percent involved only marijuana and alcohol, and 12 percent involved marijuana, alcohol, and other drug(s). Alcohol was present in nearly half of all marijuana visits that involved multiple drugs.

In terms of numbers, 4,313 ED visits by youth age 12 to 17 involved marijuana with alcohol. Marijuana also was reported frequently with cocaine (1,711), amphetamine (760), unnamed benzodiazepines (668), unnamed narcotic analgesics (626), LSD (519), and methamphetamine (444) (**Table 1**, page 4).

FIGURE 3

Marijuana-related ED visits, age 12 to 17: 1994 to 2001



These combinations appear to be changing. For youth age 12 to 17, unnamed narcotic analgesics (626), heroin (282), and unnamed tricyclic antidepressants (264) were among the top 10 drugs reported with marijuana in 2001. None of these drugs appeared in the top 20 in 1994.

Why marijuana leads to ED visits is unclear.

When multiple drugs are involved, it is not possible to know which drug (or combination) precipitated the ED visit. Only one reason for the ED visit and one motive for abusing the drug are recorded for each ED visit, regardless of the number of drugs involved.

However, the reason for the visit can be determined in cases where marijuana is the only drug. Among youth age 12 to 17 in 2001, where marijuana was the only drug reported, *psychic effects* (60%) was the most frequently cited motive for the marijuana use. *Dependence* was cited in 15 percent of cases.

Unexpected reaction (40%) was the most frequently reported reason for these ED visits. *Overdose* (10%), *chronic effects* (6%), *accident/injury* (4%), *seeking detoxification* (3%), and *withdrawal* (2%) were less frequent reasons.

These patterns differ somewhat for the multi-drug visits. For motive, multi-drug visits tended to include more suicide attempts and overdoses, with proportionately fewer psychic effects and unexpected reactions.

[6] The proportion for youth age 18 to 19 did not change; it was 31 percent in 1994 and 2001.

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TABLE 1

Top 20 drugs mentioned with marijuana in ED visits, youth age 12 to 17: 1994 and 2001

Substance mentioned with marijuana	1994
alcohol-in-combination	1,936
LSD (lysergic acid diethylamide)	783
cocaine	699
methamphetamine	528
amphetamine	276
PCP (phencyclidine)	229
acetaminophen-diphenhydramine	198
diazepam	159
datura sauveolens	126
psilocybin	114
benzodiazepines-NOS	99
anxiolytics, sedatives, and hypnotics-NOS	82
acetaminophen	77
paroxetine	76
hydrocodone	72
mescaline	70
clonazepam	69
sertraline	66
caffeine	64
amitriptyline	60

Substance mentioned with marijuana	2001
alcohol-in-combination	4,313
cocaine	1,711
amphetamine	760
benzodiazepines-NOS	668
narcotic analgesics-NOS	626
LSD (lysergic acid diethylamide)	519
methamphetamine	444
heroin	282
tricyclic antidepressants-NOS	264
PCP (phencyclidine)	252
acetaminophen	234
psilocybin	212
barbiturates-NOS	208
MDMA (Ecstasy)	198
alprazolam	181
sertraline	154
clonazepam	152
acetaminophen-chlorpheniramine	134
flunitrazepam	119
drug unknown	111

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Emergency Department Trends From the Drug Abuse Warning Network, Final Estimates 1995–2002

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TRENDS IN MAJOR SUBSTANCES OF ABUSE

This section presents annual estimates from DAWN for total drug-related ED episodes and mentions of major substances of abuse.

"Major substances of abuse" include the most common illicit drugs reported to DAWN, alcohol reported in combination with any other substance reported to DAWN, and lower frequency drugs of particular policy interest (e.g., club drugs such as MDMA (Ecstasy), and GHB). The specific terms (including street names) reported to DAWN for each drug category are listed, with corresponding mentions from 1995 to 2002, in Table 2.4.0 (full-year estimates) and Table 12.4.0 (full-year rates per 100,000 population). Corresponding half-year tables are Tables 2.3.0 and 12.3.0, respectively.

One ED episode can include mentions of one drug alone or mentions of multiple drugs. Major substances of abuse, such as cocaine, heroin, and marijuana, are often reported in combination with other drugs. Alcohol is reportable to DAWN only when present in combination with another reportable drug. Therefore, the total number of drug mentions exceeds the number of episodes. Mentions for individual drugs, which may be recorded only once per episode, are equivalent to episodes. For example, the number of cocaine mentions is equivalent to the number of episodes in which cocaine was mentioned.

The following discussion focuses primarily on comparisons of final estimates for 2002 with 2001. Tables also show statistical tests comparing 2002 estimates with those for 2000 and, for long-term trends, 2002 estimates with those for 1995 (the earliest year shown in the tables). In addition, long-term trends in drug-related ED episodes overall and for those involving the most frequently mentioned illicit drugs and alcohol-in-combination are shown in Figure 3.

DAWN estimates for 2002 are based on data from a nationally representative sample of 437 responding hospitals (Table 1.1).

TOTAL DRUG-RELATED ED EPISODES

- In 2002, there were 670,307 drug abuse-related ED episodes in the coterminous U.S., with 1,209,938 drug mentions (on average, 1.8 drugs per episode) (Table 2.2.0).
- There were no significant increases from 2001 to 2002 in total drug-related ED episodes or in ED drug mentions (Table 2.2.0). Total ED visits (that is, ED visits for any reason) increased 2 percent (from 100.5 million to 102.8 million) during this period.
- In 2002, drug abuse-related ED visits occurred at the rate of 261 ED episodes per 100,000 population in the coterminous U.S. (Table 12.2.0).
- From 2001 to 2002, among the most common major substances of abuse, only amphetamines showed a significant increase (17%, from 18,555 to 21,644). Mentions of alcohol-in-combination (207,395 in 2002), cocaine (199,198), marijuana (119,472), heroin (93,519), and methamphetamine (17,696), were all statistically unchanged. There were no significant decreases among these substances (Table 2.2.0).

- Among the less frequently mentioned major substances of abuse, only 2 had significant increases from 2001 to 2002. Mentions of inhalants rose 187 percent (from 522 to 1,496), returning to the level observed in 2000, and mentions of PCP rose 25 percent (from 6,102 to 7,648). Mentions of LSD decreased (-68%, from 2,821 to 891). MDMA (Ecstasy) (4,026), GHB (3,330), miscellaneous hallucinogens (1,428), and Ketamine (260), were statistically unchanged from 2001 to 2002. Mentions of flunitrazepam (Rohypnol) and illicit combinations NTA were too imprecise for publication (Table 2.2.0).
- Among the major substances of abuse, the highest rates of ED drug mentions in 2002 occurred for the following substances (Table 12.2.0):
  - Alcohol-in-combination (81 mentions per 100,000 population),
  - Cocaine (78),
  - Marijuana (47), and
  - Heroin (36).

ALCOHOL-IN-COMBINATION

- Alcohol-in-combination was mentioned in 31 percent of ED drug episodes in 2002 (207,395 mentions) and remains the most common substance reported in drug-related ED visits (Table 2.2.0 and Figure 3). Alcohol is reported to DAWN only when present in combination with another reportable drug, so the actual number of alcohol-related ED visits is higher than the DAWN estimate for alcohol-in-combination episodes.
- Mentions of alcohol-in-combination were statistically unchanged from 2001 to 2002, but have increased 24 percent (from 166,897 mentions) since 1995 (Table 2.2.0 and Figure 3).

COCAINE, HEROIN, MARIJUANA

- Cocaine continues to be the most frequently mentioned illicit substance, present in 30 percent of ED episodes (199,198 mentions) in 2002. Cocaine was followed in frequency by marijuana (18%, 119,472 mentions), and heroin (14%, 93,519 mentions) (Table 2.2.0 and Figure 3).
- Cocaine, heroin, and marijuana mentions were statistically unchanged from 2001 to 2002 (Table 2.2.0).
- About one-fifth of the cocaine mentions in 2002 (21%, 42,146 mentions) were attributed to "crack." This number has been stable since 1995. Most cocaine mentions (78%, 155,381) were reported to DAWN simply as "cocaine," and it is not possible to determine what proportion of these might be crack. Mentions that were reported simply as "cocaine" increased 54 percent from 1995 to 2002 (from 101,043 to 155,381), but did not increase from 2000 to 2002, or 2001 to 2002 (Table 2.4.0).
- Taking changes in population into account, from 1995 to 2002, cocaine mentions increased 33 percent (from 58 to 78 mentions per 100,000 population). Also during

this period, heroin mentions increased 22 percent (from 30 to 36), and marijuana mentions increased 139 percent (from 19 to 47) (Table 12.2.0).

AMPHETAMINES AND METHAMPHETAMINE

- In 2002, amphetamines and methamphetamine were each mentioned in 3 percent of drug abuse-related ED episodes (21,644 mentions of amphetamines; 17,696 mentions of methamphetamine) (Table 2.2.0). Only rarely were they reported together in the same ED visit, and it is not possible to know the accuracy of distinctions between them. Most mentions of amphetamines (93%) are reported simply as "amphetamine," while methamphetamine mentions are most frequently identified as "methamphetamine" (66%) or "speed" (13%) (Table 2.4.0). Together amphetamines and methamphetamine accounted for 39,340 mentions in 2002.

- From 1995 to 2002, mentions of amphetamines increased 126 percent (from 9,581 to 21,644), and the rate of amphetamine mentions increased 105 percent (from 4 to 8 mentions per 100,000 population). From 2001 to 2002, mentions of amphetamines rose 17 percent (from 18,555), and the rate of mentions of amphetamines increased 15 percent (from 7 to 8 mentions) (Table 12.2.0). Methamphetamine mentions were statistically unchanged from 2001, 2000, or 1995. This stability masks a period of great fluctuation in methamphetamine ED mentions during the late 1990s.

CLUB DRUGS

- No significant changes from 2001 to 2002 were evident for the club drugs MDMA (Ecstasy) (4,026 mentions in 2002), GHB (3,330), or Ketamine (260) (Table 2.2.0).

- The percentage changes in MDMA and GHB mentions from 1995 to 2002 are very large because of very small numbers in 1995 (Table 2.2.0). Both drugs remain relatively infrequent in ED visits, with no more than 2 mentions per 100,000 population in 2002 (Table 12.2.0).

- Estimates for flunitrazepam (Rohypnol) have been too imprecise for publication every year from 1995 through 2002 (Table 2.2.0 and Figure 4).

OTHER TRENDS

- Among the less frequently mentioned major substances of abuse (Table 2.2.0):
  - Mentions of inhalants increased 187 percent (from 522 in 2001 to 1,496 in 2002), returning to the level observed in 2000.
  - Mentions of PCP increased 25 percent (from 6,102 to 7,648) from 2001 to 2002.
  - Mentions of LSD continued to decline, with a 68 percent decrease from 2001 to 2002 (from 2,821 to 891).
  - No significant changes were evident for miscellaneous hallucinogens from 2001 to 2002 (from 1,788 to 1,428).

- For the 15 major substances of abuse (displayed in Figure 4), relative standard errors (RSEs) in 2002 range from a low of 10.0 for alcohol-in-combination to a high of 78.9 for combinations NTA. Any DAWN estimate with an RSE exceeding 50 percent is considered too imprecise for publication and is therefore suppressed in the tables. In 2002, estimates for methamphetamine, Ketamine, miscellaneous hallucinogens, flunitrazepam (Rohypnol), GHB, inhalants, and combinations NTA all had RSEs greater than 20 percent. Only the RSE for flunitrazepam (Rohypnol) exceeded 50 percent (66%) (Table RSE-2.4.0).

Figure 3
ED drug-related episodes and alcohol-in-combination, cocaine, heroin, and marijuana mentions: 1995 through 2002

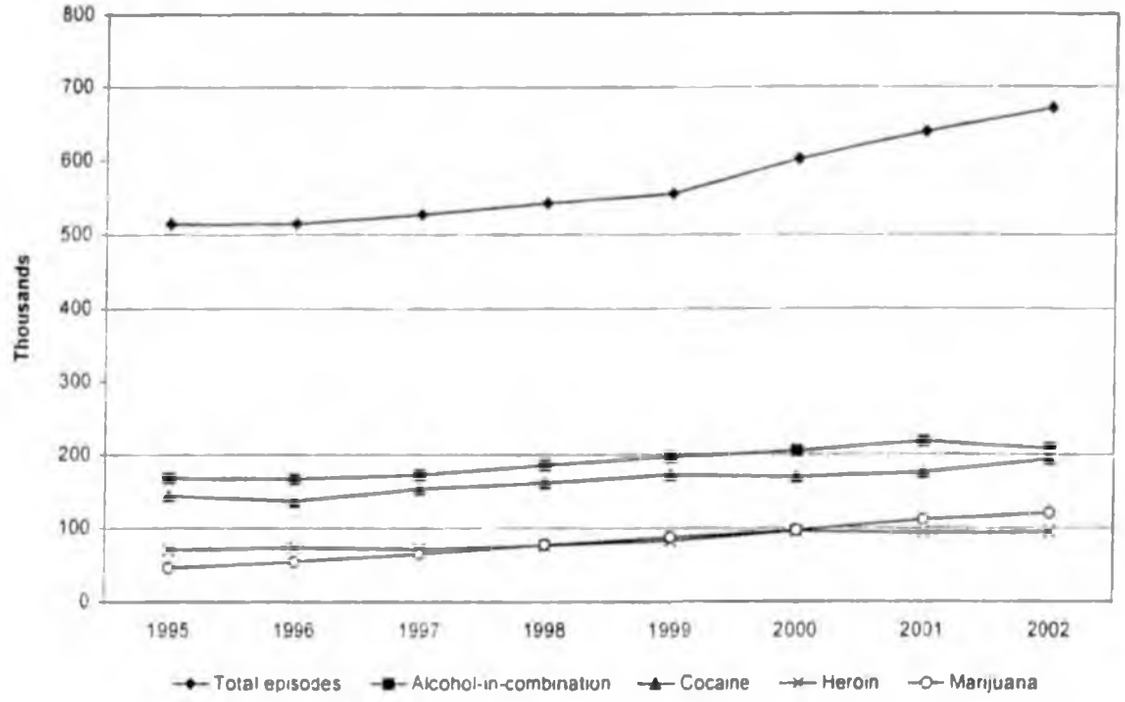
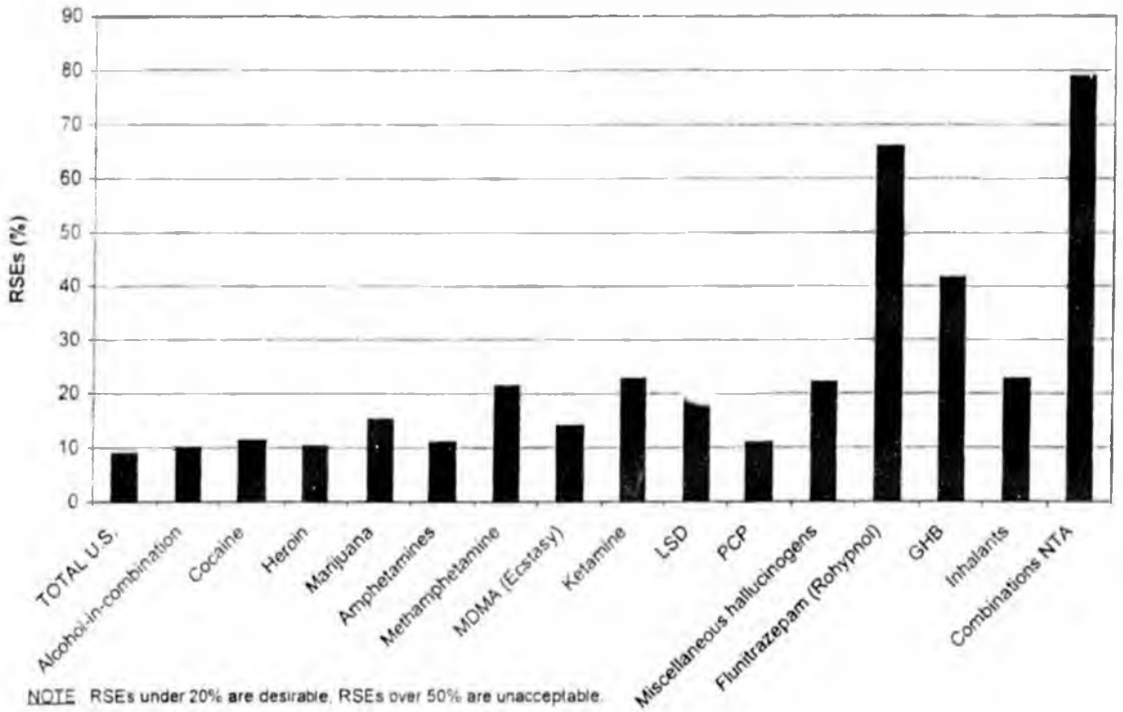


Figure 4
Relative standard errors (RSEs) for major substances of abuse: 2002



NOTE: RSEs under 20% are desirable. RSEs over 50% are unacceptable.

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TRENDS IN OTHER SUBSTANCES OF ABUSE

DAWN also receives reports of ED episodes involving the nonmedical use of legal drugs. These can involve deliberate abuse of prescribed or legally obtained over-the-counter (OTC) medications or of pharmaceuticals diverted for abuse. Accidental overdoses or adverse reactions to OTC or prescription drugs taken as directed are not reportable to DAWN unless they were present in combination with an illicit drug.

These "other substances of abuse" are tabulated first by categories composed of similar substances (Tables 2.2.0 and 12.2.0 for full-year estimates and rates, respectively) and then by generic drug name for the largest categories: psychotherapeutic agents (Tables 2.6.0 and 12.6.0), central nervous system (CNS) agents (Tables 2.8.0 and 12.8.0), respiratory agents (Tables 2.10.0 and 12.10.0), and cardiovascular agents (Tables 2.12.0 and 12.12.0). Corresponding half-year tables (2.5.0, 2.7.0, 2.9.0 and 2.11.0 for estimates and 12.5.0, 12.7.0, 12.9.0 and 12.11.0 for rates) are reported in this publication as well.

By design, all drug mentions in DAWN are tabulated either as major substances of abuse or as other substances of abuse. There is no double counting, and the deliberate assignment of drugs into major substances is the result of specific interest in such substances.

Drugs are presented in DAWN publications by generic names (e.g., acetaminophen, rather than Tylenol), and DAWN estimates should not be attributed to drugs marketed under particular brand (trade) names. DAWN data are extracted from medical records produced in the course of health care delivery (no patient is ever interviewed), so DAWN case reports contain information about particular substances as that information was documented in the ED medical record. Any prescription or OTC drug may be reported to DAWN by its brand (trade) name, generic name, or chemical name, depending on what was documented in the source record. There is no way to discern whether the brand names in the medical record are always accurate or how frequently particular brands might have been recorded by generic name. Therefore, brand names are recoded into generic names, and we do not publish estimates by brand. An index linking brand to generic names is available online at http://DAWNinfo.samhsa.gov/. The index is provided solely as an aid to readers who may be unfamiliar with generic names.

This discussion focuses mainly on comparisons of estimates from 2001 to 2002.

OTHER SUBSTANCES OF ABUSE

- DAWN estimates that other substances of abuse (527,981 mentions) comprised 44 percent of total ED drug mentions in 2002 (Table 2.2.0). Although the vast majority of these other substances are marketed legally by prescription or OTC, it is impossible to know from DAWN the number of ED visits related to the abuse of prescription drugs by patients with a legitimate prescription.

- ED mentions of other substances of abuse in 2002 were most concentrated in 2 categories—CNS agents (227,342 mentions) and psychotherapeutic agents (223,481

mentions)—in nearly equal proportions (19% and 18% of total ED mentions, respectively) (Table 2.2.0).

- The particular drugs involved in ED visits are sometimes unknown or unknowable. In 2002, there were 30,544 such mentions (3% of total mentions) (Table 2.2.0).

PSYCHOTHERAPEUTIC AGENTS

- Overall, mentions of psychotherapeutic agents were statistically unchanged from 2001 to 2002 (Table 2.2.0).
- Mentions of psychotherapeutic agents were up 9 percent since 2000 (from 204,527 to 223,481) and 18 percent since 1995 (from 190,270) (Table 2.2.0).
- Psychotherapeutic agents in DAWN are broken into 4 subcategories: antidepressants; antipsychotics; anxiolytics, sedatives, and hypnotics; and CNS stimulants.

Antidepressants

- Antidepressants (5% of total ED mentions, 62,635 mentions) were the second most frequent psychotherapeutic agents mentioned in drug-related ED visits in 2002, and as a category have remained statistically unchanged in recent years (Table 2.2.0). This category includes:
  - MAO inhibitors (14 mentions),
  - SSRI antidepressants (27,914),
  - Tricyclic antidepressants (11,546), and
  - Miscellaneous antidepressants (23,161).

MAO Inhibitors

- From 1995 to 2002, mentions of MAO inhibitors overall decreased 95 percent (from 303 to 14), but no significant change was evident from 2000 to 2002, or from 2001 to 2002 (Table 2.6.0).

SSRI Antidepressants

- From 1995 to 2002, mentions of SSRI antidepressants overall increased 29 percent (from 21,585 to 27,914), but no significant change was evident from 2000 to 2002 (Table 2.6.0).
- From 2001 to 2002, no significant changes were evident for any of the SSRI antidepressants. In 2002, the most frequently mentioned SSRIs (Table 2.6.0) were:
  - Citalopram (5,313 mentions), which rose 54 percent from 2000 to 2002,
  - Fluoxetine (5,770), down 39 percent from 1995 to 2002, and down 27 percent from 2000 to 2002,

- Paroxetine (9,443), up 67 percent from 1995 to 2002, and
- Sertraline (7,214), which has remained relatively stable in recent years.

Tricyclic Antidepressants

- Overall, mentions of tricyclic antidepressants decreased 41 percent (from 19,429 to 11,546) from 1995 to 2002, but have remained stable in the last 3 years (Table 2.6.0).
- From 2001 to 2002, no significant changes were evident for any of the SSRI antidepressants, and from 2000 to 2002, only amitriptyline mentions changed significantly. In 2002, the most frequently mentioned tricyclic antidepressants (Table 2.6.0) were:
  - Amitriptyline (4,436 mentions), down 50 percent from 1995 and down 31 percent from 2000 to 2002,
  - Doxepin (868), down 68 percent since 1995,
  - Imipramine (242), down 90 percent since 1995,
  - Nortriptyline (424), down 82 percent since 1995, and
  - Tricyclic antidepressants not identified by name (noted as "not otherwise specified" or "-NOS") (5,397), with no change from 1995, 2000 or 2001.

Miscellaneous Antidepressants

- Overall, mentions of miscellaneous antidepressants increased 86 percent (from 12,447 to 23,161) from 1995 to 2002, but remained stable from 2000 to 2002, and from 2001 to 2002 (Table 2.6.0).
- Among the miscellaneous antidepressants, only venlafaxine mentions changed significantly during the 3 years from 2000 to 2002. In 2002, the category of miscellaneous antidepressants (Table 2.6.0) included:
  - Buproprion (4,074 mentions), up 226 percent since 1995,
  - Mirtazapine (2,222),
  - Nefazodone (923), up 294 percent since 1995,
  - Trazadone (9,560),
  - Venlafaxine (5,501), up 345 percent since 1995, up 48 percent since 2000, and up 38 percent since 2001; and
  - Unnamed antidepressants (antidepressants-NOS) (875), up 508 percent from 1995 to 2002.

Antipsychotics

- Mentions of substances classified as antipsychotics were statistically unchanged from 1995, 2000 and 2001. In 2002, this category included 4 subcategories, but more than 90 percent of mentions fell into the single subcategory of miscellaneous antipsychotic agents.

- In 2002, there were 18,492 ED mentions of miscellaneous antipsychotic agents. This estimate was statistically unchanged from 2000 but 67 percent higher than in 1995 (Table 2.6.0). However, the trends for the individual antipsychotic agents in this category varied considerably; they include:

  - Haloperidol (911 mentions), down 67 percent since 1995,
  - Lithium (2,231), down 67 percent since 1995, down 40 percent since 2000, and down 35 percent since 2001,
  - Olanzapine (4,207), unchanged over the periods 1995, 2000 and 2001 to 2002,
  - Quetiapine (6,508), up 116 percent since 2000 and up 50 percent since 2001, and
  - Risperidone (3,566), up 248 percent since 1995.

- Other significant long-term trends in antipsychotics included thioridazine which declined 98 percent (from 2,566 to 48 mentions), fluphenzaine, which declined 95 percent (from 792 to 42), prochlorperazine, which declined 66 percent (from 555 to 191), and chlorpromazine, which declined 64 percent (from 2,202 to 795 mentions) from 1995 to 2002 (Table 2.6.0). Thioridazine also significantly decreased from 2000 to 2002, down 94 percent from 782.

Anxiolytics, Sedatives, and Hypnotics

- Anxiolytics, sedatives, and hypnotics (137,350, or 11% of total ED mentions) were the most frequent psychotherapeutic agents mentioned in drug-related ED visits in 2002 (Table 2.2.0). This category includes 3 subcategories, none of which posted significant changes from 2001 to 2002:

  - Barbiturates (1%, 9,783 mentions), with an increase of 38 percent from 2000 to 2002,
  - Benzodiazepines (9%, 105,752), with a 16 percent increase from 2000 to 2002, and
  - Miscellaneous anxiolytics, sedatives, and hypnotics (2%, 21,816), which were statistically unchanged since 2000.

Barbiturates

- From 2001 to 2002, ED mentions of the barbiturates, individually and as a class, were statistically unchanged (Table 2.2.0).

- From 1995 to 2002, barbiturate mentions rose 44 percent (from 6,793 to 9,783) (Table 2.2.0).

- In 2002, the most frequently mentioned barbiturates were unnamed (barbiturates-NOS, with 7,579 mentions) (Table 2.6.0). Mentions of barbiturates-NOS increased 56 percent from 2000 to 2002, and 110 percent from 1995 to 2002.
- Phenobarbital, the second most frequently mentioned barbiturate in 2002 with 1,217 mentions, decreased 58 percent from 1995 to 2002 (Table 2.6.0).

Benzodiazepines

- In 2002, mentions of benzodiazepines (105,752) accounted for 9 percent of all ED drug mentions. Overall, mentions of benzodiazepines increased 16 percent (from 91,078) from 2000 to 2002 (Table 2.2.0 and Figure 5). Since 1995, mentions of benzodiazepines have risen 38 percent (from 76,548).
- From 2001 to 2002, ED mentions of the benzodiazepines, individually and as a class, were statistically unchanged.
- In 2002, the most frequently mentioned benzodiazepines (Table 2.6.0 and Figure 6) were:
  - Alprazolam (27,659 mentions),
  - Clonazepam (17,042),
  - Diazepam (11,193),
  - Lorazepam (11,042),
  - Temazepam (2,219), and
  - Unnamed benzodiazepines (i.e., benzodiazepines-NOS, 34,697).
- From 1995 to 2002, among the most frequently mentioned benzodiazepines (Table 2.6.0 and Figure 6):
  - Mentions of benzodiazepines-NOS rose 199 percent, alprazolam rose 62 percent, and clonazepam 33 percent, while
  - Mentions of diazepam, lorazepam, and temazepam remained stable.
- From 2000 to 2002, all the benzodiazepines except alprazolam and benzodiazepines-NOS were statistically unchanged (Table 2.6.0).
  - Mentions of alprazolam rose 25 percent (from 22,105 to 27,659),
  - Mentions of benzodiazepines-NOS increased 55 percent (from 22,376 to 34,697).
- Mentions of 2 of the less frequently mentioned benzodiazepines decreased from 1995 to 2002 (Table 2.6.0):
  - Chlordiazepoxide (-74%, from 2,661 to 696), and
  - Triazolam (-77%, from 776 to 175).