0022-3565/94/2683-1612$00.00/0
THE JOURNAL OF PHARMACOLOGY AND EXPERIMENTAL THERAPEUTICS

Vol. 268, No. 3
Printed in U.S.A.

Localization of Cannabinoid Receptors and Nonsaturable High-Density Cannabinoid Binding Sites in Peripheral Tissues of the Rat: Implications for Receptor-Mediated Immune Modulation by Cannabinoids

ALLISON B. LYNN and MILES HERKENHAM
Section on Functional Neuroanatomy, National Institute of Mental Health, Bethesda, Maryland
Accepted for publication October 27, 1993

ABSTRACT

[^{3}H]CP-55,940, a high-affinity cannabinoid receptor ligand, was used for *in vitro* binding and autoradiography in peripheral tissues in the rat. Specific cannabinoid receptor binding was found to be restricted to components of the immune system, *i.e.*, spleen, lymph nodes and Peyer's patches. Displacement studies showed that this binding is identical (similar K_d and structure-activity profile) to that in brain. Cannabinoid receptors in the immune system are confined to B lymphocyte-enriched areas, *i.e.*, the marginal zone of the spleen, cortex of the lymph nodes and nodular corona of Peyer's patches. Specific binding is absent in T lymphocyte-enriched areas, such as the thymus and periarteriolar lymphatic sheaths of the spleen. Certain macrophage-enriched areas, *i.e.*, liver and lung, lack specific binding. Thus, the single peripheral cell type that may contain cannabinoid receptors is the B lymphocyte. Numerous sites have dense binding that could not be displaced by excess unlabeled drug. These nonspecific sites were found in the liver, adrenal glands and sebaceous glands, which are high in fat content, and in the heart, pancreas, components of the male and female reproductive systems and the epithelium of the esophagus. Thus, the highly lipophilic nature of cannabinoids does not appear to be the sole determinant of nonspecific binding. The data suggest that cannabinoids may exert specific receptor-mediated actions on the immune system of rats. Perhaps, also at high concentrations, cannabinoids exert membrane effects at sites where they are sequestered nonspecifically.

In the centuries since marijuana (*Cannabis sativa*) was first used as a psychoactive drug, the most significant discoveries in regard to its mechanism of action have been made only recently, with the isolation of Δ^9-THC as the principal active ingredient (Mechoulam *et al.*, 1970), the characterization and localization of the cannabinoid receptor in the brain (Devane *et al.*, 1988; Herkenham *et al.*, 1990), the cloning of its gene (Matsuda *et al.*, 1990), and the identification of an endogenous ligand (Devane *et al.*, 1992). Recent work also shows that most cannabinoid effects are receptor mediated (Herkenham *et al.*, 1990, Howlett *et al.*, 1988) and appear to occur through CNS action (Martin, 1986).

In addition to the well known CNS effects, peripheral actions of cannabinoids have been implicated; these include actions on the male reproductive tract (reduced spermatogenesis and altered sperm morphology; Hembree *et al.*, 1991; Patra and Wadsworth, 1990), the immune system (specific immunosuppression; Arata *et al.*, 1992; Cabral and Vasquez, 1992; Friedman, 1991; Kaminski *et al.*, 1992; Klein and Friedman, 1986; Nahas *et al.*, 1974; Pross *et al.*, 1990; Specter *et al.*, 1991; Specter *et al.*, 1991; White *et al.*, 1975), the endocrine system (suppression of prolactin and follicle-stimulating hormone; Block *et al.*, 1991; and estradiol; Murphy *et al.*, 1991), the digestive tract (inhibition of ileal contractions; Nye *et al.*, 1985; Rosell and Agurell, 1975; and antiemesis; Bhargava, 1978), and the cardiopulmonary system (tachycardia in humans; Beaconsfield *et al.*, 1972; Clark, 1975; bradycardia in animals; Dewey, 1986; and bronchodilation; Bernstein *et al.*, 1976; Bhargava, 1978). Of these effects, immune suppression (Kaminski *et al.*, 1992) and guinea pig ileal contractions have been scrutinized by structure-activity studies (Nye *et al.*, 1985; Rosell and Agurell, 1975); the results suggest the existence of peripheral receptors in these organs. Other actions may be mediated by CNS receptors (*e.g.*, endocrine effects, antiemesis and cardiac changes) but a peripheral site of action cannot be excluded.

Received for publication May 10, 1993.

ABBREVIATIONS: BSA, bovine serum albumin; CNS, central nervous system; CP-55,940/CP-55,667, (*cis*)-3-[2-hydroxy-4-(1,1 dimethylheptyl)phenyl]-(*trans*)-4-(3-hydroxypropyl)cyclohexanol; GNP-PNP, guanylyl-imidodiphosphate; H&E, hematoxylin and eosin; PBS, phosphate-buffered saline; mRNA, messenger RNA; RT, room temperature; THC, tetrahydrocannabinol.

To date, there is limited evidence for cannabinoid receptors in discrete peripheral tissue. Cannabinoid receptor binding and mRNA have been found in mouse spleen (Kaminski *et al.*, 1992), and cannabinoid receptor mRNA has been identified in human testes (Gérard *et al.*, 1991). However, another mechanism by which cannabinoids might affect peripheral organ and tissue physiology is through high-dose nonspecific membrane effects. Cannabinoids are extremely lipophilic and are stored for long periods in fat (Kreuz and Axelrod, 1973). Other areas of high concentration after *in vivo* administration are the liver, heart, lung, kidney, and spleen (Agurell *et al.*, 1986; Bronson *et al.*, 1984; Ryrfeldt and Ramsay, 1973). In these organs, cannabinoids may be sequestered in the fat and/or the cellular membranes.

In this study, the binding of [^{3}H]CP-55,940, a synthetic radiolabeled cannabinoid with a high potency and high specific activity, was used to localize cannabinoid receptor binding in the peripheral tissue of rats. Both total and nonspecific binding were measured to assess the relative contributions of specific (receptor total bound − nonspecific bound) and nonspecific (an *in vitro* index of lipophilia, deposition and sequestration) binding in each tissue. In tissues with specific binding, displacement studies that used known cannabinoids of various potencies (which included enantiomeric pairs) were carried out to compare affinities and relative potencies of binding at peripheral receptors *versus* central receptors. Nonspecific binding was evaluated and compared with patterns of *in vivo* distribution of cannabinoids in the body (Bronson *et al.*, 1984; Ryrfeldt and Ramsay, 1973). Sites of high concentration *in vitro* and *in vivo* may be sites where cannabinoids exert high-dose nonspecific effects on organ physiology (Dax *et al.*, 1989; Hembree *et al.*, 1991; Patra and Wadsworth, 1990).

Materials and Methods

Ten rats (Sprague-Dawley, five male and five female) were used. Of the 10, 6 were deeply anesthetized with chloral hydrate and pentobarbital so that structures from the neck region (thyroid gland, trachea and esophagus, lymph nodes and submaxillary and parotid glands) could be dissected out and unclotted blood could be collected by cardiac puncture. The remaining four animals were decapitated unanesthetized. Dissected organs were frozen in 2-methyl butane at −30°C or on aluminum foil on dry ice, depending on size, then mounted on chucks and cryostat cut at −20°C. Series of 15 μm-thick serial sections were collected on gelatin-coated slides. Adjacent sections were used for binding (total and nonspecific). The sections were briefly dried at 30°C and stored at −35°C until they were processed. A third series of adjacent sections was fixed in formaldehyde vapors (Herkenham, 1988), delipidated, and stained with H&E.

Blood was collected by cardiac puncture into 2-ml Vacutainers (Becton Dickinson, Rutherford, NJ) that contained EDTA. After a 10-min centrifugation at 3000 rpm, the buffy coat was carefully pipetted off, washed with PBS and centrifuged three subsequent times (10 min, 3000 rpm) to concentrate white blood cells. Aliquots (25 μl) were smeared onto gelatin-coated slides, dried at RT and stored at −35°C until processing. Equivalent smears were vapor fixed and stained with H&E for cell counting.

Crude extract of bone marrow was obtained by removing the pelvis and femur from the rat, crushing the bone with rongeurs to reach the marrow and placing it in 3 ml of ice-cold PBS. The extract was then centrifuged at 3000 rpm for 10 min. Bony spicules sedimented first and the cell containing layer pelleted above. Supernatant was removed and replaced with 500 μl of PBS to resuspend the cell-containing layer, which was smeared in 25-μl aliquots onto gelatin-coated slides, dried at RT and stored at −35°C until processing. Equivalent smears were vapor fixed and stained with H&E.

Binding was carried out as described previously (Herkenham *et al.*, 1991). The binding parameters used were those optimized for brain sections. Because we did not fully characterize the peripheral binding, it can only be assumed that equilibrium conditions were approximated.

For sections from proteolytic, acidic or lipotryptic structures (stomach, pancreas, intestine and cecum), a 30-min preincubation in binding buffer was used. Brain sections were treated in parallel to verify that no binding was lost during the preincubation. Incubation of 10 nM [^{3}H]CP-55,940 (New England Nuclear, Boston, MA; custom labeled, 79 Ci/mmol) was for 2.5 hr at 37°C in 50 mM Tris (pH 7.4)/5% BSA binding buffer. We added 10 μM CP-55,244 (all CP analogs were obtained from Pfizer, Groton, CT; the most potent cannabinoid in the CP series) to assess nonspecific binding in adjacent sections. Sections were washed for 4 hr at 0°C in 50 mM Tris (pH 7.4)/1% BSA buffer (2 rinses). After washing, sections were dipped in 50 mM Tris buffer (pH 7.4)/0.5% formaldehyde at RT (5 min) to remove excess BSA and preserve tissue integrity, dipped briefly (5 sec) in water, dried under a cool stream of air, placed in cassettes, and apposed to tritium-sensitive film (^{3}H-Hyperfilm, Leica, Arlington Heights, IL) for 10 days.

For drug-displacement studies, serial sections from structures with high specific binding were incubated in 10 nM [^{3}H]CP-55,940 in the presence of 5-fold serial dilutions (this covered 10^4 concentration range) of known active and inactive cannabinoids: (−)-Δ^9-THC, (+)-Δ^9-THC, cannabidiol, 11-(OH)-Δ^9-THC (a potent THC metabolite; THC analogs were obtained from the National Institute on Drug Abuse, Rockville, MD), CP-55,940, CP-56,667 (a nonpsychoactive enantiomer of CP-55,940), nabilone (obtained from Lilly Inc.), and GMP-PNP (an allosteric inhibitor of CP-55,940 binding in brain, obtained from Boehringer-Mannheim, Indianapolis, IN). Concentrations of the displacers were selected to bracket their K_i values previously derived in brain (Herkenham *et al.*, 1990, 1991). Binding was analyzed by quantitative autoradiography (described subsequently) after 10-day film exposure. Tissue equivalents of bound ligand *versus* concentration of cold drug were plotted to ascertain IC_{50} and K_i obtained by linear regression of logit/log plots and calculated with the Cheng/Prusoff equation.

Densitometric quantitation of all autoradiograms was achieved by coexposure of sections with tritium standards (high- and low-density Micro-scales, Amersham, Arlington Heights, IL). Developed films were illuminated with a light box. Images were captured with a solid-state video camera onto a Macintosh II computer-based densitometry system by using IMAGE software (Wayne Rasband, Research Services Branch, National Institute of Mental Health, Bethesda, MD). Average light transmittance values of outlined structures were converted to nanocuries per milligram of tissue (wet weight) using a best-fit polynomial equation that related transmittance levels to tissue-equivalent values of the standards. Binding was expressed as femtomoles bound per milligrams of tissue (wet weight) based on the specific activity of the ligand (79 Ci/mmol) and the decay of the standards.

Significant specific binding was assessed by one-factor analysis of variance, followed by a Dunnet two-tailed *post hoc* test that compared total *versus* nonspecific binding. By arbitrary criteria, nonspecific binding was considered significant if greater than 50 fmol/mg of wet weight (nonspecific binding in brain is typically 10–15 fmol/mg of wet weight).

Results

Control brain sections from anesthetized and unanesthetized animals showed no significant differences in binding; therefore, structures from both sets were pooled. Brain sections preincubated with lytic tissue (then incubated with the remainder of tissue) showed no differences in binding characteristics compared with "incubated only" sections; therefore, tissue-derived contaminants do not appear to reduce binding. It is still possible that intrinsic proteases present in the tissue may degrade

receptors partially (our incubation buffer does not include protease inhibitors), which caused an underestimation of specific binding observed in some tissues.

Localization of specific receptor binding. Of all the tissues examined, specific binding was always associated with components of the immune system, as described later. The total specific and percent specific binding are presented table 1.

The spleen contained the highest density of specific [^{3}H]CP-55,940 binding outside the brain. The whole spleen had about one-half the level of receptors found in the brain and the binding was 64% specific compared with 92% in the brain (table 1). Within the spleen, receptor binding was heterogeneous; the highest amount was localized to the white pulp marginal zone and perhaps also to the mantle of white pulp follicles (table 1). Although these two compartments are separated by a sheath of reticular cells seen in adjacent H&E-stained sections (fig. 1A, D), the resolution of the film image was insufficient to permit definitive assignment of receptor localization to the mantle portion of the follicles. Moderate levels of specific binding were found in the red pulp and the lowest specific binding in the spleen was seen in the central regions of the white pulp (germinal centers and periarteriolar lymphatic sheaths).

Further examination of the immune system was carried out by fine dissection of smaller lymphatic tissues. The cervical lymph nodes had moderate levels of specific binding in the cortex and background levels in the medulla and lymphatic hilus (table 1, fig. 2B). A similar pattern and level of specific binding were found in lymphatic nodules of the gut. Although intestinal sections displayed no specific binding in the mucosa, submucosa, and serosa, [^{3}H]CP-55,940 did bind moderately and specifically to Peyer's patches located in the subserosa of the jejunum, ileum, and rectum (table 1, fig. 2C). Ligand binding was high in the corona of each nodule and low in the germinal center and in areas between the nodules.

Of all structures with a predominantly immune function, the thymus was uniquely devoid of specific [^{3}H]CP-55,940 binding despite its high concentration of lymphocytes and its physiological role in T lymphocyte differentiation (table 1).

Smears of blood sample buffy coats incubated with [^{3}H]CP-55,940 had specific binding (table 1). Our method for smearing cells was crude; it was intended to maximize the number of cells (especially leukocytes) per slide by selecting for the buffy coat after the first centrifuge spin and subsequent washes. Cell counts showed a distribution of 5 to 24 $\times$ 10^6 erythrocytes per slide with approximately 2 to 7 $\times$ 10^5 white blood cells (approximately 1.5–3.5% leukocytes as opposed to the 0.15% in whole blood). Failure to disperse cells uniformly during smearing and loss of cell adhesion during the binding assay left the results with a high degree of variation; however, of slides smeared with equivalent volumes of suspended cells, the total binding was consistently higher than was the nonspecific binding (62% specific binding). After binding, the slides were vapor fixed and counterstained. Microscopic examination revealed only white blood cells remaining intact on the slide after the incubation, although membrane fragments from other cells adhering to the slide could account for the specific cannabinoid binding. In other sections that contained high concentrations of normal blood (*e.g.*, the chambers of the heart), no specific binding could be detected; therefore, leukocyte-enriched smears do appear to concentrate a cannabinoid receptor-binding component of the blood.

Because of the high degree of variability between smears, specific binding in the bone marrow was not statistically significant. However, on average, total binding was 39% higher than equivalent smears for nonspecific binding (table 1).

The structures found with significant levels of specific binding, *i.e.*, the spleen, lymph nodes, and Peyer's patches, were assayed for competitive displacement of various cannabinoids in order to determine inhibition constants (K_is). The K_i values derived from immune tissue for various cannabinoids correlated well with the psychotropic and physiological potencies of the same drugs and with K_i values determined in brain sections and neuroblastoma cell lines as shown in table 2 and reviewed by Herkenham *et al.* (1990). The correlation of brain section K_is (Herkenham *et al.*, 1990) *versus* spleen section K_i is shown in figure 3. Enantioselectivity of binding was evident in the immune tissue. Enantiomeric pairs examined were (−)-Δ^9-THC *versus* (+)-Δ^9-THC and CP-55,940 (-AC) *versus* CP-56,667 (+AC) (see Johnson and Melvin, 1986, for the chemical structures of CP compounds).

The sensitivity to guanine nucleotides was also assessed in a single experiment by using tissue sections collected from two animals. Binding in immune tissue was inhibited by 100 μM GMP-PNP, which similarly inhibits [^{3}H]CP-55,940 binding in the brain (Herkenham *et al.*, 1991). Specific binding in the spleen was reduced from 54.1 ± 7.2 fmol/mg to 31.1 ± 11.2 fmol/mg (43% reduction); in lymph nodes, from 27.2 ± 1.0 fmol/mg to 13.4 ± 1.3 fmol/mg (50% reduction); and in Peyer's patches, from 35.3 ± 1.5 fmol/mg to 15.8 ± 3.1 fmol/mg (54% reduction).

Outside the CNS, the only nonlymphoid site of specific binding was found in the anterior pituitary (25% specific binding, table 1), an observation documented earlier (Herkenham *et al.*, 1991). No other tissue had binding greater than 10% specific.

Localization of nonspecific binding. Numerous tissues displayed high levels of nonspecific (nondisplaceable) binding (table 1, fig. 4). The highest level in all tissues was found in the lining of the upper esophagus (fig. 4A). Male and female sexual organs contained high levels in complex patterns (fig. 4B–E). In male rats, a high level of nonspecific binding was found in the urogenital tract, concentrated in the epididymis (fig. 4B, C). The epithelial lining of the epididymis displayed an extremely high density of nonspecific binding, which was most robust in the lower caput (fig. 4B) and upper cauda epididymis (fig. 4C) where the epithelial cells have a characteristic tall columnar shape. It was much lower in the distal cauda epididymis where the epithelial cells flatten to a cuboidal shape. The lumen of the epididymis also displayed gradients of nonspecific binding; this was moderate where the epididymal tubules exit the testes and throughout the caput (fig. 4B) and upper cauda portions (fig. 4C) of the epididymis, and it dropped to low levels in the distal half of the cauda epididymis where the lumen enlarges before exiting to the efferent ductus deferens. Because of the marked decrease in signal in both the lumen and lining, the cauda epididymis can be compartmentalized into a "dark region" (continuous with the caput epididymis) and "light region" (continuous with the efferent ductus deferens, fig. 4C). Continuous with the tubules of the epididymis, nonspecific binding in the mucosa of the vas deferens (fig. 4C, inset) was high. The testes displayed only low-to-moderate levels of uniform binding. Tissues low in nonspecific binding

TABLE 1

Distribution of [^{3}H]CP-55,940 binding in peripheral tissues

The data are means (n = 4) and standard deviations.[a]

Structure	Total	Nonspecific	Specific	Percent Specific Binding
		fmol/mg wet weight tissue		%
Brain	157 ± 5	13 ± 2	143***	92
Immune system				
Whole spleen	92 ± 12	33 ± 7	59***	64
Red pulp	73 ± 11	29 ± 5	44**	60
Mantle/marginal zone	136 ± 17	29 ± 4	108***	79
Periarteriolar lymphatic sheaths/ germinal center	61 ± 9	30 ± 4	31**	52
Whole lymph node	44 ± 9	23 ± 5	22***	47
Lymph node cortex	53 ± 9	19 ± 6	28***	63
Lymph node medulla/hilus	27 ± 5	20 ± 7	—	—
Thymus	32 ± 9	26 ± 6	—	—
Peyer's patch (ileum, jejunum, rectum)	57 ± 10	32 ± 6	25**	43
Blood buffy coat smear	25 ± 10	9 ± 1	16*	62
Bone marrow smear	32 ± 5	19 ± 6	—	—
Endocrine glands				
Anterior pituitary gland	33 ± 4	24 ± 2	8*	25
Adrenal gland	76 ± 11	69 ± 12	—	—
Thyroid gland	26 ± 5	25 ± 4	—	—
Parathyroid gland	27 ± 5	25 ± 5	—	—
Liver	60 ± 5	64 ± 7	—	—
Heart	66 ± 8	61 ± 10	—	—
Lung	17 ± 9	12 ± 4	—	—
Digestive system				
Upper esophagus				
Epithelium	101 ± 12	89 ± 10	—	—
Lamina propria	17 ± 5	18 ± 4	—	—
Lamina muscularis	24 ± 5	25 ± 4	—	—
Lower esophagus				
Epithelium	47 ± 3	42 ± 10	—	—
Lamina propria	16 ± 3	21 ± 5	—	—
Lamina muscularis	27 ± 4	31 ± 6	—	—
Cardiac stomach				
Epithelium	42 ± 2	43 ± 2	—	—
Lamina propria/muscularis	25 ± 2	27 ± 2	—	—
Pyloric stomach	26 ± 6	32 ± 7	—	—
Pancreas	53 ± 6	48 ± 5	—	—
Duodenum	34 ± 2	32 ± 3	—	—
Jejunum	42 ± 3	40 ± 3	—	—
Ileum	31 ± 5	31 ± 5	—	—
Cecum	19 ± 5	20 ± 6	—	—
Colon	28 ± 3	27 ± 3	—	—
Rectum	31 ± 4	29 ± 3	—	—
Excretory system				
Kidney	34 ± 5	27 ± 7	—	—
Bladder	17 ± 5	15 ± 8	—	—
Female reproductive system				
Ovary	47 ± 7	47 ± 7	—	—
Mature follicles	74 ± 17	75 ± 17	—	—
Oviduct	22 ± 5	20 ± 4	—	—
Uterus				
Epithelium	40 ± 12	40 ± 13	—	—
Lamina propria	13 ± 3	15 ± 3	—	—
Lamina muscularis	20 ± 4	18 ± 4	—	—
Vagina				
Epithelium	89 ± 41	75 ± 37	—	—
Lamina propria/muscularis	14 ± 4	14 ± 3	—	—
Male reproductive system				
Testes	37 ± 7	40 ± 6	—	—
Epididymis				
Caput epididymis	90 ± 7	92 ± 6	—	—
Cauda epididymis				
Dark region	108 ± 13	105 ± 15	—	—
Light region	53 ± 7	51 ± 6	—	—
Seminal vesicles	22 ± 4	22 ± 3	—	—
Prostate gland	40 ± 2	40 ± 3	—	—
Bulbourethral gland	25 ± 4	24 ± 6	—	—

TABLE 1 Continued

Structure	Total	Nonspecific	Specific	Percent Specific Binding
	fmol/mg wet weight tissue			%
Vas deferens				
Epithelium	91 ± 17	86 ± 17	—	—
Lamina propria/muscularis	42 ± 8	39 ± 9	—	—
Skeletal muscle	33 ± 3	33 ± 4	—	—
Visual structures				
Retina	35 ± 6	29 ± 4	—	—
Ciliary body	11 ± 4	11 ± 5	—	—
Secretory structures				
Exopthalmic lacrimal gland	30 ± 3	29 ± 3	—	—
Harder's gland	41 ± 4	41 ± 4	—	—
Gland of Meibom (eyelid)	82 ± 36	69 ± 32	—	—
Submaxillary gland	22 ± 3	22 ± 4	—	—
Parotid gland	32 ± 5	31 ± 5	—	—

[a] Significance established by a one-factor analysis of variance and a Dunnet two-tailed *post hoc* test comparing total and nonspecific binding.
* $P \leq .05$.
** $P \leq .001$.
*** $P \leq .0001$.

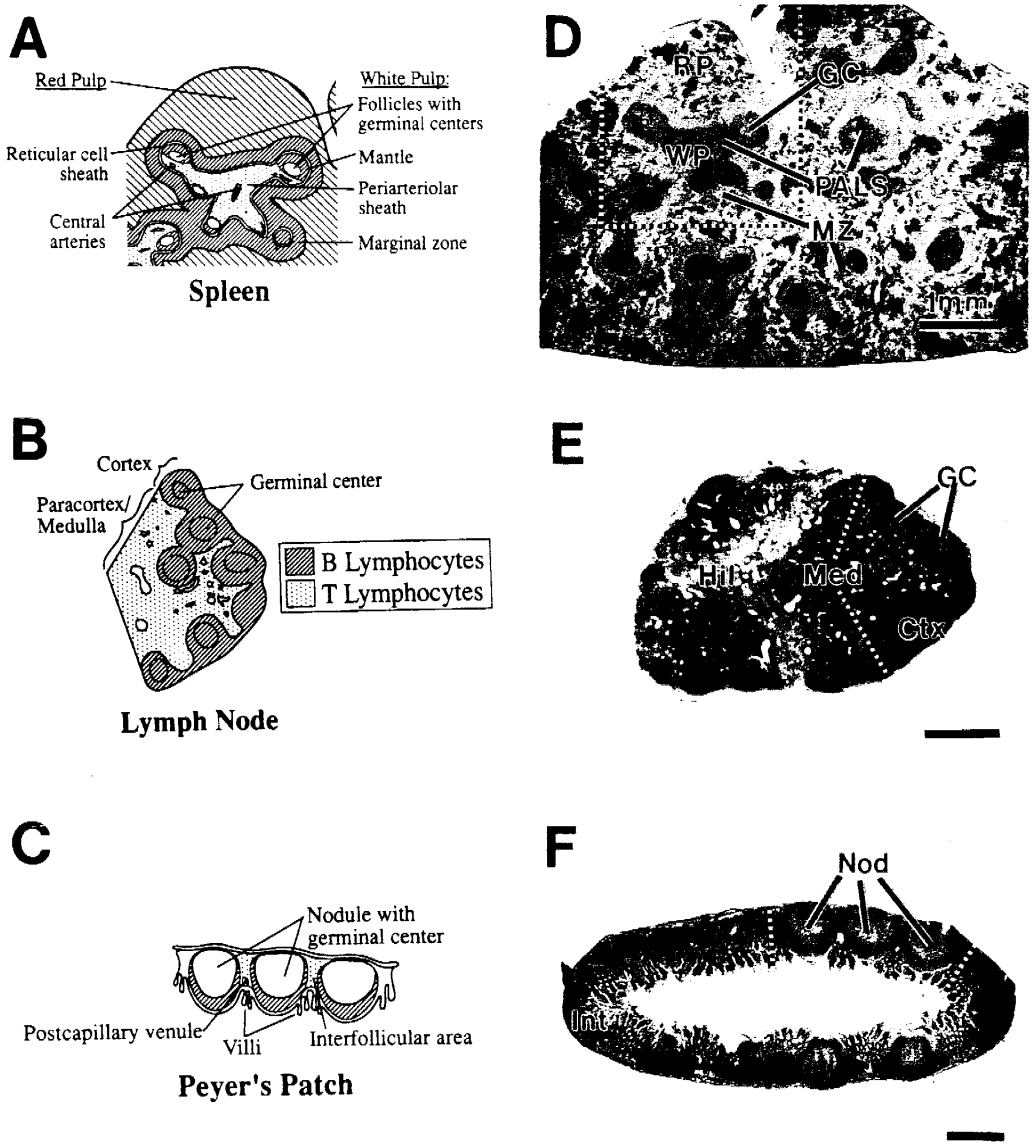

Fig. 1. Diagrammatic representation of B and T lymphocyte-enriched areas in immune tissue (modified from Carter and Bazin, 1973). Areas diagrammed in more detail (A–C) are outlined on stained sections (D–F) and in ensuing autoradiograms of [^{3}H]CP-55,940 binding (fig. 2A–F). A and D) Region of stained section of the spleen compartmentalized into red pulp (RP) and white pulp (WP) and containing periarteriolar lymphatic sheaths (PALS) around central arteries; splenic follicles of the white pulp contain pale-staining germinal centers (GC) and an outer mantle (not easily seen). Also, components of the white pulp are a thin reticular cell layer between the PALS and the marginal zone (MZ). B and E) A central section of a cervical lymph node with the hilus (Hil), medulla (Med) and Cortex (Ctx), which contains the germinal centers (GC). C and F) Peyer's patches (lymphatic nodules, Nod) in the submucosa of the intestine (Int). The bar equals 1 mm.

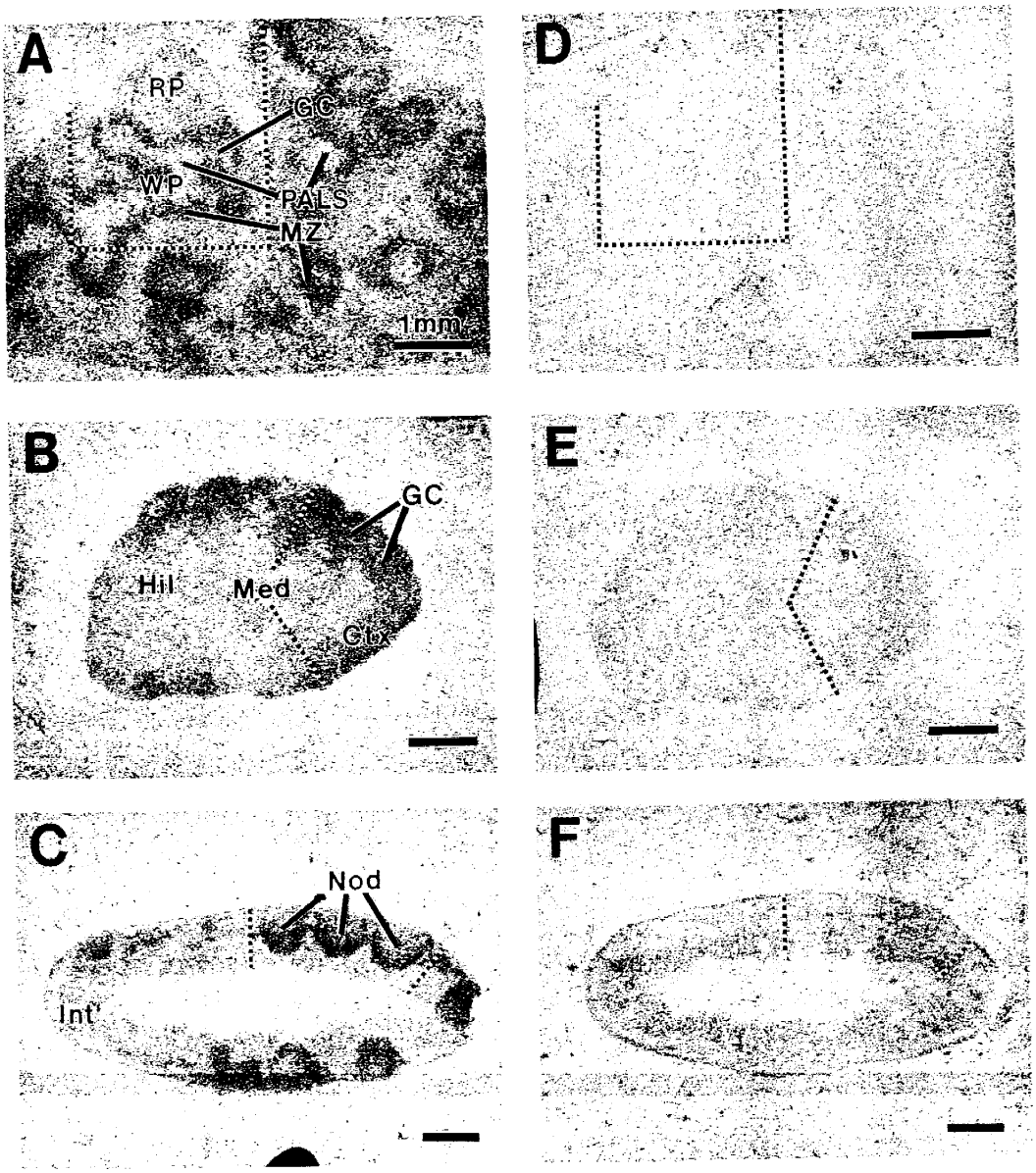

Fig. 2. Cannabinoid receptors in immune tissue. A) In the spleen, [^{3}H]CP-55,940 binding is highest in the marginal zone and, possibly, mantle layers of splenic follicles in the white pulp (WP). It is low in the periarteriolar lymphatic sheaths (PALS) around central arteries and moderate in the red pulp (RP). B) In the lymph node, significant specific binding is isolated to the cortex (Ctx), which contains the germinal centers (GC), and is absent in the medulla (Med), paracortex (T cell areas) and hilus (Hil). C) Specific binding in the gut is found only in Peyer's patches of the intestine (Int), where binding is dense in the corona of each nodule (Nod) and sparse in internodular areas and germinal centers. D–F) Nonspecific binding to tissue in the presence of 1000-fold greater concentration of CP-55,244. The bar equals 1 mm.

TABLE 2

Binding inhibition constants of cannabinoids in immune tissue and brain

The data are in means ± S.E.M.

Compound	Brain K_i*	Spleen K_i (n = 2)	Lymph Node K_i (n = 4)	Peyer's Patch K_i (n = 4)
		nM		
CP 55,244	1.4	0.80 ± 0.48	2.4 ± 0.51	0.44 ± 0.14
CP 55,940 (−ACD)	15	7.2 ± 2.4	3.8 ± 0.6	4.0 ± 1.3
CP 55,667 (+ACD)	470	160 ± 160	430 ± 150	540 ± 370
Nabilone	120	300 ± 190	1010 ± 130	790 ± 50
11-OH-Δ^9-Tetrahydrocannabinol	210	200 ± 100	410 ± 30	120 ± 50
(−)-Δ^9-Tetrahydrocannabinol	420	700 ± 8	480 ± 270	720 ± 190
(+)-Δ^9-Tetrahydrocannabinol	7700	13,300 ± 1600	9700 ± 3200	17,800 ± 4700
Cannabidiol	53,000	41,800 ± 3200	38,100 ± 9900	34,500 ± 14,500

* Data from Herkenham *et al.*, 1990.

were the seminal vesicles and coagulating gland, the prostate gland, and the bulbourethral gland (table 1).

Of the female reproductive tissues, the highest nonspecific [^{3}H]CP-55,940 binding occurred in the vaginal epithelium (fig. 4D) and in ovarian follicles in which the binding density correlated with the maturity of each follicle. Binding was low in the remainder of the ovary that contained immature follicles and hilus and the attached oviduct (fig. 4E). The uterus showed low-density nonspecific binding in the lamina muscularis and a slightly higher density in the uterine epithelium (table 1).

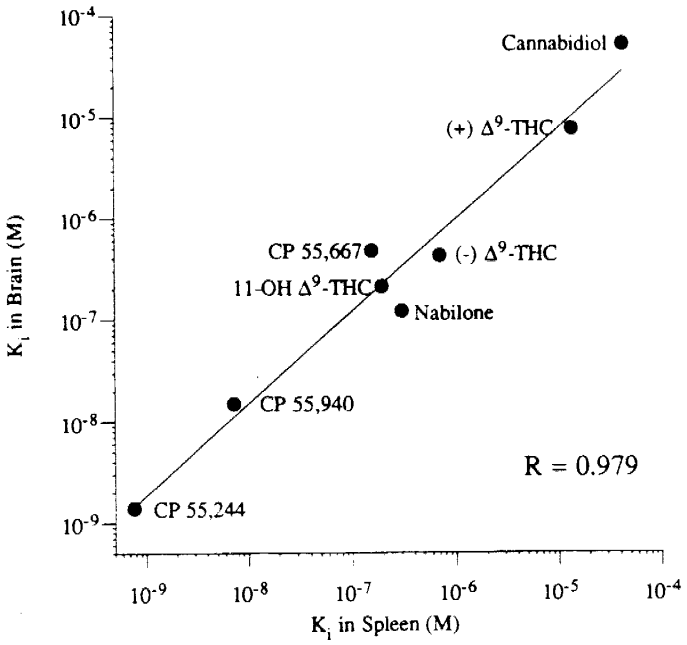

Fig. 3. Structure-activity analysis of cannabinoid displacement from receptors in spleen *versus* brain (Herkenham *et al.*, 1990) shows a strong correlation of binding K_is. Similar K_is for cannabinoids were determined in lymph node and Peyer's patch section binding assays (table 2). Note the enantioselectivity of receptors reflected in the binding affinities of (−)- and (+)-Δ^9-THC and CP-55,940 and its enantiomer CP-55,667.

Nonspecific binding of [^{3}H]CP-55,9540 was low in the bladder, urethra, and medulla of the kidney of both male and female rats. A low-to-moderate density of binding was observed in the cortex of the kidney (table 1).

In gastrointestinal tissue, there was a tendency for the mucosal layer to display higher densities of nonspecific binding. This trend was most apparent in the upper esophagus and throat, where the squamous epithelium had an extremely high density of nonspecific binding (fig. 4A), especially by comparison with the surrounding muscle, the density of which was close to the film's background level. This density also followed a gradient, with nonspecific binding in the esophageal epithelium near the stomach being low (table 1). In the cardiac portion of the stomach, the density of the inner mucosa was slightly higher than that in the outer serosa/muscularis, but the two layers had equally low levels in the pyloric portion. All other tissue of the gut (duodenum, ileum, jejunum, cecum, colon, and rectum) showed low nonspecific binding (with the exception of the aforementioned specific binding in Peyer's patches, table 1).

The heart displayed a moderate-to-high degree of uniform nonspecific binding. Also, high nonspecific binding could be visualized in fatty tissue attached to the heart (fig. 4G). High levels in the cardiac muscle were remarkable by comparison with lower levels of nonspecific binding in other muscle types, namely, skeletal (moderate) and visceral smooth muscle (low). Of similar density to the heart was the liver and pancreas (fig. 3H, I, respectively). Respiratory structures (trachea and lung) had low levels of nonspecific binding.

The endocrine organs examined included the adrenal, thyroid and parathyroid glands. Nonspecific binding was elevated only in the adrenal cortex (fig. 4F).

Several secretory structures were dissected or were included in larger structures sectioned. Nonspecific binding was moderate to low in the submaxillary and parotid salivary glands and the exophthalmic lacrimal gland. Dissected with the eye were the ciliary body supplying the anterior chamber, the harderian gland supplying the nictitating membrane, and the sebaceous tarsal glands supplying the eyelid. Nonspecific binding in the harderian gland was moderate to high and, in the tarsal gland, it was very high, probably reflecting fat content. The ciliary body had an extremely low binding density.

Discussion

Whereas most of the known physiological effects of cannabinoids have been attributed to actions initiated by binding to CNS receptors, a large body of scientific and historical literature exists that indicates that cannabinoids can also act directly on peripheral target organs, either specifically (receptor mediated) or nonspecifically (possibly through membrane actions; Martin, 1986). Such "side effects" of cannabinoids include cardiovascular alterations (Beaconsfield *et al.*, 1972; Clark, 1975; Dewey, 1986), pulmonary effects (Bernstein *et al.*, 1976; Bhargava, 1978), immunosuppression (Arata *et al.*, 1992; Cabral and Vasquez, 1992; Friedman, 1991; Klein and Friedman, 1986; Nahas *et al.*, 1974; Pross *et al.*, 1990; Specter *et al.*, 1991; Specter *et al.*, 1991; White *et al.*, 1975), reproductive dysfunction (Hembree *et al.*, 1991; Gérard *et al.*, 1991; Patra and Wadsworth, 1990) and endocrine modulation (Block *et al.*, 1991; Dax *et al.*, 1989; Murphy *et al.*, 1991). Δ^9-THC and its analogs have proposed clinical efficacy in the treatment of glaucoma because of cannabinoid-induced decreases in intraocular pressure (Bhargava, 1978; Adler and Geller, 1986; Merritt, 1982) and for antiemesis (Bhargava, 1978). Some of the effects on peripheral tissue physiology are centrally mediated, whereas other effects may be the result of direct specific and/or nonspecific actions on peripheral tissue. The present study attempts to identify potential peripheral sites of action by localizing both specific and nonspecific *in vitro* [^{3}H]CP-55,940 binding. The results indicate that, outside the brain, cannabinoid receptors are scarce and confined in small numbers to components of the immune system. However, nonspecific binding, which is low and uniform in the brain, is plentiful and heterogeneously distributed in peripheral tissues and deserves its own commentary (discussed subsequently).

Specific binding to peripheral cannabinoid receptors. The evidence for a close linkage between the brain and immune system is extensive. Immune tissues are richly innervated by the sympathetic and parasympathetic neurons. In addition, neuropeptide receptors (*e.g.*, opiate, substance P, vasoactive intestinal peptide, and neuropeptide Y) are in abundance in immune tissues, such as the spleen, thymus, lymph nodes, and lymphatic nodules (Siglinga and Goldstein, 1988; Stanisz *et al.*, 1987; Wiedermann *et al.*, 1986). Immunohistochemical studies have revealed peptidergic neural innervation in these tissues (Fink and Weihe, 1988; Kendall and Al-Shawaf, 1991; Ottaway *et al.*, 1987). Thus, neuronally active substances are often also immunomodulatory and it is not unexpected that an exhaustive survey of peripheral cannabinoid receptor localization would reveal receptor populations in immune tissue with similar binding characteristics to those in the brain. Although probably not peptidergic, current evidence indicates that the endogenous cannabinoid ligand is an arachidonic acid metabolite (Devane *et al.*, 1992), which suggests eicosanoid modulation of immune responses.

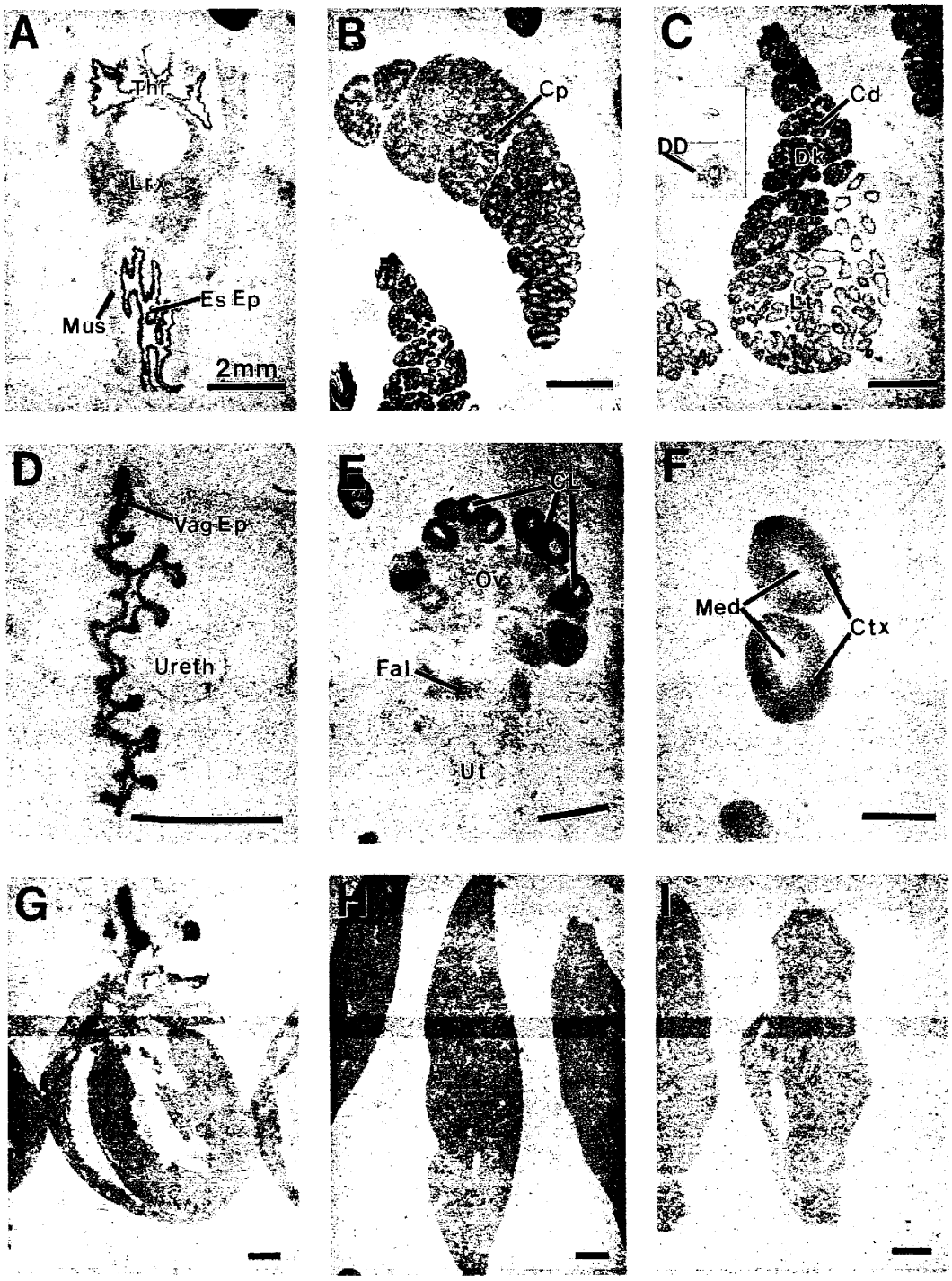

Fig. 4. Tissues containing high levels of nonspecific binding. Despite inclusion of 10 μM CP-55,244 in the incubation, [^{3}H]CP-55,940 binding was high in several areas. In sections of the throat (A), including the trachea, esophagus, thyroid and parathyroid, the epithelium of the upper throat (Thr) and esophagus showed the highest degree of nonspecific binding. In the male rat, high nonspecific binding was found in the both the cauda (B) and caput (C) epididymis and in the epithelium of the ductus deferens (DD, inset in C). In the cauda epididymis, binding followed a gradient from a dark (Dk) to light (Lt) in the distal end, paralleling a transition from tall columnar to low columnar epithelial cells and the widening of the epididymal tubule before exit into the ductus deferens epididymis. Nonspecific binding was high in the vaginal epithelium of the female rat (D) and low in the adjacent urethra (Ureth). In the ovary (Ov), the density was high in the outer mature follicles (Fol) and low in maturing follicles and in the hilus of the ovary (E). The density was also low in the fallopian tubes (Fal) and the uterus (Ut). In the adrenal gland (F), binding was high in cortex (Ctx) and low in medulla (Med). Larger organs that displayed moderate-to-high nonspecific binding were the heart (G), liver (H) and pancreas (I). The bar equals 2 mm.

Unlike the other well characterized CNS receptors in the periphery, cannabinoid receptors are noteworthy for being confined to the immune system. Within the immune system, cannabinoid receptors appear in the spleen, Peyer's patches, lymph nodes, bloods, and bone marrow. Receptors do not appear in the thymus, liver, or lung. The resolution of the technique is insufficient to permit a definitive determination of the specific cell types within the receptor-bearing immune organs. However, considering the binding patterns, we can speculate that the predominant cell type that bears cannabinoid receptors is the B lymphocyte.

Specific binding in the spleen (fig. 2A) is densest in the outermost layers of white pulp, the mantle layers of splenic follicles, and the marginal zone. These regions are highly invested with lymphocytes and, according to mapping studies of lymphocyte subtypes in rat (Goldschneider and McGregor, 1973), they contain predominantly B cells (fig. 1A). Specific binding is moderate in the red pulp and sparse in the most central periarteriolar lymphatic sheaths (considered a T lymphocyte domain) and germinal centers of the white pulp.

Specific [^{3}H]CP-55,940 binding in the lymph nodes is localized to the cortex. Analogous to the high-density pattern in the spleen, this distribution matches that of B lymphocytes and not T lymphocytes (Goldschneider and McGregor, 1973). T cells are localized to the paracortex and medulla where receptor binding is sparse (figs. 1B, 2B).

In Peyer's patches, specific receptor binding is localized in the corona of each nodule (figs. 1C, 2C), which suggests a B lymphocyte origin because B cells are most concentrated in the nodular corona, whereas T cells are described as migrating to the internodular spaces (Carter and Bazin, 1973), a site of unremarkable binding density.

Given the evidence to date of cannabinoid modulation of T lymphocyte activity (Gupta *et al.*, 1974; Nahas *et al.*, 1974; Pross *et al.*, 1990), it is an unexpected finding that cannabinoid receptor binding was absent in the T cell-enriched areas of the spleen (periarteriolar lymphatic sheath of the spleen, medulla of lymph nodes and internodular spaces of Peyer's patches). Especially striking was the complete absence of detectable specific binding in the thymus, a site of T cell differentiation (although it contains for the most part immature T cells).

Macrophages cannot be ruled out as a possible binding component in the spleen because areas high in binding also contain these phagocytes. In the marginal zone of the spleen, where specific [^{3}H]CP-55,940 binding is densest, macrophages are abundant: these cells are the marginal metallophyls, a distinct subgroup of the reticuloendothelial system. Moderate levels of specific binding in the red pulp of the spleen may also reside on a phagocytic cell population. Here the venous sinuses and splenic cords are heavily invested with macrophages. Also, specific binding is present in leukocyte-enriched blood smears. Monocytes/macrophages are adherent to glass slides and therefore would be the most likely cells that remain after hypotonic ligand binding. However, a microscopic examination of the intact cells that remained on the slides after binding, washing, autoradiographic processing, and counter staining showed the presence of both species of leukocytes—monocytes and lymphocytes. Therefore, one population could not be singled out as the binding component.

There is strong evidence of Δ^9-THC-induced inhibition of macrophage function (Arata *et al.*, 1992; Barbers *et al.*, 1991; Cabral and Vasquez, 1991; Lopez-Cepero *et al.*, 1986; Specter *et al.*, 1991). Whether such macrophage modulation is receptor mediated could be addressed more directly with binding studies on pure cultured macrophages. Evidence to the contrary of macrophages as a binding component for [^{3}H]CP-55,940 in this study included the absence of specific binding in other macrophage-containing structures, *e.g.*, lung (alveolar macrophages) and liver (Kupffer cells).

Cannabinoid receptors are coupled to an inhibitory subunit of G proteins, as indicated by substantial cannabinoid suppression of adenylate cyclase activity in neuroblastoma cells (Howlett, 1985). The inhibition of specific binding by GMP-PNP in the present study confirmed this coupling in peripheral tissues. Inhibition of cellular function within the immune realm includes an *in vitro* reduction of macromolecular synthesis and mitogen-induced lymphocyte proliferation (Klein *et al.*, 1985; Pross *et al.*, 1990), suppression of macrophage burst characteristics in marijuana smokers (Sherman *et al.*, 1991), and suppression of other macrophage extrinsic and intrinsic functional parameters *in vitro* (phagocytosis, cell spreading, susceptibility to infection, and conferring of antiviral activity on cocultured cells) (Barbers *et al.*, 1991; Lopez-Cepero *et al.*, 1986; Specter *et al.*, 1991). It is not known which, if any, of these effects is receptor mediated. The present data support cannabinoid receptor localization to B lymphocytes and, perhaps, also to macrophages (but only in certain compartments of the immune system).

To date, evidence has emerged for cannabinoid effects on B cells. *In vitro* inhibition of lipopolysaccharide-induced B cell proliferation of mouse splenocytes by Δ^9-THC was more profound than suppression of T proliferation (Klein and Friedman, 1986). Recently, Kaminski *et al.* (1992) showed localization of cannabinoid receptors in the spleen by using a membrane binding assay and, although they were unable to identify the cell type responsible for binding, they showed profound inhibition of antibody production (a predominantly B cell task) in splenocytes by CP-55,940.

If cannabinoids do modulate B cell migration, proliferation, plasma cell morphogenesis, and immunoglobulin production, such effects may have profound implications for the humoral immune response. Whereas suppression of B cell-mediated function could be detrimental (although there is little evidence that it is *in vivo*), cannabinoid suppression could play a positive therapeutic role in autoimmune disorders. Lyman *et al.* (1989) showed that Δ^9-THC is an effective prophylactic treatment for autoimmune encephalomyelitis (an animal model for multiple sclerosis) in Lewis rats and strain-13 guinea pigs, and they proposed a mechanism whereby lymphocyte migration and sequestration in the CNS are inhibited by the drug. Only subtle immune suppression has been noted in humans (Gross *et al.*, 1991; Nahas *et al.*, 1974; Sherman *et al.*, 1991), which suggests a species difference in peripheral cannabinoid receptor distributions and densities.

Species differences may also explain the absence of specific receptor binding in two areas of rat that would appear to have receptors in other species on the basis of functional assays and structure-activity studies. In excised guinea pig ileum, the potencies of cannabinoids to inhibit ileal contractions (Nye *et al.*, 1985; Rosell and Agurell, 1975) correlated well with potencies in structure-activity analyses of psychopotency, dog static ataxia, adenylate cyclase inhibition, mouse analgesia, and brain receptor binding (Herkenham *et al.*, 1990). Similarly, in mouse vas deferens, inhibition of contractions was a reliable assay for

cannabinoid potencies (Devane *et al.*, 1992). However, there was no detectable receptor binding found in the rat ileum or vas deferens in our assay. Sections of guinea pig ileum and mouse vas deferens should therefore be examined for specific cannabinoid receptor binding.

Nonspecific binding. Sites of high nonspecific binding deserve consideration in evaluating the fate of the drug after *in vivo* administration. Sequestration of high levels of cannabinoids in lipophilic membranes may allow drug-membrane interactions of the type that occur with high (micromolar) concentrations of drug. After *in vivo* administration of [^{3}H]Δ^9-THC to animals, the resulting tissue distribution of radioactivity is not a pattern of receptor distribution (the ligand has a low potency and low specific activity) but, rather, drug and drug metabolite deposition and sequestration in tissues are determined by blood flow, perfusion rate, and lipid content. The pattern of nonspecific *in vitro* CP-55,940 binding in the present study reflected the *in vivo* Δ^9-THC distribution pattern to some degree, with especially good correspondence of dense label in fatty tissues. However, differences between the *in vivo* and the *in vitro* patterns were also noted as a result of factors that make the two preparations different, namely, metabolism and the elimination of the drug *in vivo* and poor adherence of fat to slides *in vitro*.

High *in vitro* binding in the liver corresponded to high levels sequestered there *in vivo* (Bronson *et al.*, 1984; Ryrfeldt and Ramsay, 1973). The liver is the primary organ of metabolism, and bile is the chief conduit of excretion (Garrett and Hunt, 1977).

High levels of nonspecific binding were seen in body fat associated with sectioned tissues. Quantitation of binding in fat is impossible because of the difficulty in sectioning fatty tissue and because most fat does not remain on the slide during processing. Nevertheless, structures with high fat content (liver, adrenal gland and tarsal gland of the eyelid) had high levels of nonspecific binding *in vitro*, which corresponded to high levels of sequestered [^{3}H]Δ^9-THC *in vivo* (Kreuz and Axelrod, 1973).

Other factors besides lipophilia of cannabinoids must dictate the sites of nonspecific *in vitro* binding because several of the tissues found to have the highest levels of nonspecific binding were not noted for their high fat content. The squamous epithelium of the upper esophagus is low in fat yet it contained the highest level of nonspecific binding common to both sexes of the rat. Characterizing possible high-affinity nonreceptor components of binding (*e.g.*, to myelin basic protein; Nye *et al.*, 1988) may answer questions in regard to the cell types susceptible to deposition of high levels of cannabinoids and cell membrane perturbations as a result of these drugs.

The heart displays a relatively high uniform density of nonspecific binding. Cardiac muscle is not high in fat yet it has a unique affinity for nonspecific [^{3}H]CP-55,940 binding by comparison with other muscle types. Such binding would appear to be without consequence because cannabinoid effects on heart rate and blood pressure (Beaconsfield *et al.*, 1972; Bhargava, 1978; Clark, 1975) can be attributed to a combination of CNS effects, such as alterations in vagus tone and modulation of the sympathetic and parasympathetic control of vascular beds (Clark, 1975).

High uniform nonspecific binding was also seen in the rat pancreas, another area not notably high in fat. High-dose nonspecific cannabinoid effects on arachidonic acid release were illustrated in the exocrine pancreas (Chaudhry *et al.*, 1988).

In the male rat, the site with the highest level of nonspecific binding was the epididymis, notably the columnar epithelium of the epididymal tubules. Binding in the lumen of the epididymis may be to the sperm localized there, as suggested by the presence of sperm in the lumen of adjacent stained sections. However, the light region of binding, immediately before the efferent vas deferens, had a low luminal signal despite a high concentration of sperm in stained sections. The different binding densities in the epididymis may reflect stages of sperm maturation in their passage through the tubules, or the luminal binding may be unrelated to sperm.

Cannabinoids *in vivo* have been implicated in aberrant sperm morphology in animals (Patra and Wadsworth, 1990) and men (Hembree *et al.*, 1991). A common feature of these studies is the high concentrations of drugs or long dosing regimens required to illicit morphological changes, implying a nonreceptor-mediated mechanism of action on sperm, perhaps within the epididymis. Reports of impaired spermatogenesis (Patra and Wadsworth, 1990) would not appear to be mediated at the level of the testes, given the lack of binding there. Cannabinoid effects on spermatogenesis and testosterone secretion suggest actions at the level of the hypothalamus, where cannabinoids inhibit luteinizing hormone-releasing hormone secretion (Dewey, 1986).

In female rats, nonspecific binding was highest in the squamous epithelium of the vagina and the ovaries, especially in the outermost mature follicles (fig. 4D, E). Evidence for high levels of unsaturable binding in mature follicles poses questions in regard to cannabinoid effects on egg morphology and fertilizability, ovarian hormone regulation and teratogenic potential. In a study by Nogawa *et al.* (1984), moderate doses of Δ^9-THC administered to mice resulted in higher incidence rates of abnormal ova after human chorionic gonadotropin treatment. However, to date, cannabinoids have not been shown to affect fertility adversely or to be teratogens, except in extremely high chronic doses (Dewey, 1986). Such effects in part may be mediated at the level of the pituitary or brain, given the evidence about prolactin suppression in frequent female users of marijuana (Block *et al.*, 1991; Dax *et al.*, 1989; Murphy *et al.*, 1991).

Summary. Besides the nervous system, cannabinoid receptors herein were localized to certain tissues of the immune system in the rat. High-density high-affinity enantioselective binding sites are found in lymphatic tissue. Whereas this finding has implications for human immunomodulation, it should be noted that rodents are more susceptible than humans to immunomodulation by cannabinoids, perhaps reflecting the substantially higher levels of receptor in their immune cells. In humans, immune suppression is subtle (Cushman and Khurana, 1976; Dax *et al.*, 1989; Friedman, 1991; Gross *et al.*, 1991; Kagen *et al.*, 1983; Nahas *et al.*, 1974; Pillai *et al.*, 1991) and in many cases insignificant (Pillai *et al.*, 1991; White *et al.*, 1975). There is little evidence for cannabinoid immunosuppression as a causative agent in disease.

Evidence from this study implicates the B lymphocyte as the principal cannabinoid receptor-bearing cell in the spleen, lymph node, and Peyer's patch, and it may also be the specific binding component in the circulating blood of the rat. T lymphocytes are unlikely sites for receptors, as evidenced by low receptor densities in the T cell-rich areas of these immune

tissues and, especially, by the absence of binding in the thymus. Macrophages cannot be discounted and may contribute to binding; however, in other structures of the reticuloendothelial system besides the spleen, specifically the liver and lung, which have large numbers of macrophages, there is no specific receptor binding.

Although the CNS psychopotency and non-CNS peripheral side effects of marijuana use under both controlled and natural settings have been noted for decades, the first step in isolating the mechanism of each effect is the separation of nonreceptor-mediated from receptor-mediated actions. This has been made possible by localizing specific and nonspecific binding of [^{3}H] CP-55,940 in peripheral tissues. Evidence for high-density nonspecific sites poses new questions as to other mechanisms by which cannabinoids may alter organ function, specifically male and female reproduction.

Note Added in Proof

A recent study (Munro *et al.*, 1993) describes the existence and localization of a distinct peripheral cannabinoid receptor gene, which is expressed in the marginal zone of the spleen, the same location that shows specific receptor binding, and in macrophages/monocytes purified by cell sorting.

References

ADLER, M. W. AND GELLER, E. B.: Ocular effects of cannabinoids. *In* Cannabinoids as Therapeutic Agents, ed. by R. Mechoulam, pp. 51–70, CRC Press, Boca Raton, FL, 1986.

AGURELL, S., HALLDIN, M., LINDGREN, J., OHLSSON, A., WIDMAN, M., GILLESPIE, H. AND HOLLISTER, L.: Pharmacokinetics and metabolism of Δ^1-tetrahydrocannabinol and other cannabinoids with emphasis on man. Pharmacol. Rev. **38:** 21–43, 1986.

ARATA, S., NEWTON, C., KLEIN, T. AND FRIEDMAN, H.: Enhanced growth of *Legionella pneumophila* in tetrahydrocannabinol-treated macrophages. Proc. Soc. Exp. Biol. Med. **199:** 65–67, 1992.

BARBERS, R. G., EVANS, M. J., GONG, H. AND TASHKIN, D. P.: Enhanced alveolar monocytic phagocyte (macrophage) proliferation in tobacco and marijuana smokers. Am. Rev. Respir. Dis. **143:** 1092–1095, 1991.

BEACONSFIELD, P., GINSBERG, J. AND RAINSBURY, R.: Marijuana smoking: Cardiovascular effects in man and possible mechanisms. N. Engl. J. Med. **287:** 209–212, 1972.

BERNSTEIN, J. G., KUEHNLE, J. C. AND MENDELSON, J. H.: Medical implications of marijuana use. Am. J. Drug Alcohol Abuse **3:** 347–361, 1976.

BHARGAVA, H. N.: Potential therapeutic applications of naturally occurring and synthetic cannabinoids. Gen. Pharmacol. **9:** 195–213, 1978.

BLOCK, R. I., FARINPOUR, R. AND SCHLECHTE, J. A.: Effects of chronic marijuana use on testosterone, luteinizing hormone, follicle stimulating hormone, prolactin and cortisol in men and women. Drug Alcohol Depend. **28:** 121–128, 1991.

BRONSON, M., LATOUR, C. AND NAHAS, G.: Distribution and disposition of delta-9-tetrahydrocannabinol (THC) in different tissues of the rat. *In* The Cannabinoids: Chemical, Pharmacological, and Therapeutic Aspects, ed. by S, Agurell, W. L. Dewey and R. E. Willette, pp. 309–317, Academic Press, New York, 1984.

CABRAL, G. A. AND VÀSQUEZ, R.: Marijuana decreases macrophage antiviral and antitumor activities. *In* Advances in the Biosciences, ed. by G. G. Nahas and C. Latour, vol. 80, pp. 93–105, Pergamon Press, New York, 1991.

CABRAL, G. A. AND VÀSQUEZ R.: Δ^9-Tetrahydrocannabinol suppresses macrophage extrinsic antiherpesvirus activity. Proc. Soc. Exp. Biol. Med. **199:** 255–263, 1992.

CARTER, P. B. AND BAZIN, H.: Immunology. *In* The Laboratory Rat, ed. by H. J. Baker, J. R. Lindsay and S. H. Weisbroth, pp. 181–212, Academic Press, New York, 1973.

CARTER, P. B. AND BAZIN H.: Immunology. *In* The Laboratory Rat, ed. by H. J. Baker, J. R. Lindsey and S. H. Weisbroth, pp. 181–212, Academic Press, New York, 1980.

CHAUDHRY, A., THOMPSON, R. H., RUBIN, R. P. AND LAYCHOCK, S. G.: Relationship between Δ^9-tetrahydrocannabinol-induced arachidonic acid release and secretagogue-evoked phosphoinositide breakdown and Ca^{2+} mobilization of exocrine pancreas. Mol. Pharmacol. **34:** 543–548, 1988.

CLARK, S. C.: Marijuana and the cardiovascular system. Pharmacol. Biochem. Behav. **3:** 299–306, 1975.

CUSHMAN, P. AND KHURANA, R.: Marijuana and T lymphocyte rosettes. Clin. Pharmacol. Ther. **19:** 310–317, 1976.

DAX, E. M., PILOTTE, N. S., ADLER, W. H., NAGEL, J. E. AND LANGE, W. R.: The effects of 9-ene-tetrahydrocannabinol on hormone release and immune function. J. Steroid Biochem. **34:** 263–70, 1989.

DEVANE, W. A., DYSARZ, F. A. I., JOHNSON, M. R., MELVIN, L. S. AND HOWLETT, A. C.: Determination and characterization of a cannabinoid receptor in rat brain. Mol. Pharmacol. **34:** 605–613, 1988.

DEVANE, W. A., HANUS, L., BREUER, A., PERTWEE, R. G., STEVENSON, L. A., GRIFFIN, G., GIBSON, D., MANDELBAUM, A., ETINGER, A. AND MECHOULAM, R.: Isolation and structure of a brain constituent that binds to the cannabinoid receptor. Science (Washington D.C.) **258:** 1946–1949, 1992.

DEWEY, W. L.: Cannabinoid pharmacology. Pharmacol. Rev. **38:** 151–178, 1986.

FINK, T. AND WEIHE, E.: Multiple neuropeptides in nerves supplying mammalian lymph nodes: Messenger candidates for sensory and autonomic neuroimmunomodulation? Neurosci. Lett. **90:** 39–44, 1988.

FRIEDMAN, H.: Cannabis and immunity. *In* Advances in the Biosciences, ed. by G. G. Nahas and C. Latour, vol. 80, pp. 79–92, Pergamon Press, New York, 1991.

GARRETT, E. R. AND HUNT, C. A.: Pharmacokinetics of Δ^9-tetrahydrocannabinol in dogs. J. Pharmacol. Sci. **66:** 395–406, 1977.

GÉRARD, C. M., MOLLEREAU, C., VASSART, G. AND PARMENTIER, M.: Molecular cloning of a human cannabinoid receptor which is also expressed in testis. Biochem. J. **279:** 129–134, 1991.

GOLDSCHNEIDER, I. AND MCGREGOR, D. D.: Anatomical distribution of T and B lymphocytes in the rat. Development of lymphocyte-specific antisera. J. Exp. Med. **138:** 1443–1465, 1973.

GROSS, G., ROUSSAKI, A., IKENBERG, H. AND DREES, N.: Genital warts do not respond to systemic recombinant interferon alpha-2a treatment during cannabis consumption. Dermatologica **183:** 203–207, 1991.

GUPTA, G., GREICO, M. AND CUSHMAN, P.: Impairment of rosette-forming T lymphocytes in chronic marijuana smokers. N. Engl. J. Med. **291:** 874–876, 1974.

HEMBREE, W. C. I., NAHAS, G. G., ZEIDENBERG, P. AND HUANG, H. F. S.: Changes in human spermatozoa associated with high dose marijuana smoking. *In* Advances in the Biosciences, ed. by G. G. Nahas and C. Latour, vol. 80, pp. 67–78, Pergamon Press, New York, 1991.

HERKENHAM, M.: Influence of tissue treatment on quantitative receptor autoradiography. *In* Molecular Neuroanatomy, ed. by F. W. van Leeuwen, R. M. Buijs, C. W. Poole and O. Pach, pp. 111–119, Elsevier, Amsterdam, 1988.

HERKENHAM, M., LYNN, A. B., JOHNSON, M. R., MELVIN, L. S., DE COSTA, B. R. AND RICE, K. C.: Characterization and localization of cannabinoid receptors in rat brain: A quantitative *in vitro* autoradiographic study. J. Neurosci. **11:** 563–583, 1991.

HERKENHAM, M., LYNN, A. B., LITTLE, M. D., JOHNSON, M. R., MELVIN, L. S., DE COSTA, B. R. AND RICE, K. C.: Cannabinoid receptor localization in brain. Proc. Natl. Acad. Sci. U. S. A. **87:** 1932–1936, 1990.

HOWLETT, A. C.: Cannabinoid inhibition of adenylate cyclase: Biochemistry of the response in neuroblastoma cell membranes. Mol. Pharmacol. **27:** 429–436, 1985.

HOWLETT, A. C., JOHNSON, M. R., MELVIN, L. S. AND MILNE, G. M.: Nonclassical cannabinoid analgetics inhibit adenylate cyclase: Development of a cannabinoid receptor model. Mol. Pharmacol. **33:** 297–302, 1988.

JOHNSON, M. R. AND MELVIN, L. S.: The discovery of nonclassical cannabinoid analgetics. *In* Cannabinoids as Therapeutic Agents, ed. by R. Mechoulam, pp. 121–145, CRC Press, Boca Raton, FL, 1986.

KAGEN, S. L., KURUP, V. P., SOHNLE, P. G. AND FINK, J. N.: Marijuana smoking and fungal sensitization. J. Allergy Clin. Immunol. **71:** 389–93, 1983.

KAMINSKI, N. E., ABOOD, M. E., KESSLER, F. K., MARTIN, B. R. AND SCHATZ, A. R.: Identification of a functionally relevant cannabinoid receptor on mouse spleen cells that is involved in cannabinoid-mediated immune modulation. Mol. Pharmacol. **42:** 736–742, 1992.

KENDALL, M. D. AND AL-SHAWAF, A.: Innervation of the rat thymus gland. Brain Behav. Immun. **5:** 9–28, 1991.

KLEIN, T. W. AND FRIEDMAN, H.: Modulation of murine immune cell function by marijuana components. *In* Drugs of Abuse and Immune Function, ed. by R. R. Watson, pp. 87–111. CRC Press, Boca Raton, FL, 1986.

KLEIN, T. W., NEWTON, C. A., WIDEN, R. AND FRIEDMAN, H.: The effect of delta-9-tetrahydrocannabinol and 11-hydroxy-delta-9-tetrahydrocannabinol on T-lymphocyte and B-lymphocyte mitogen responses. J. Immunopharmacol. **7:** 451–466, 1985.

KREUZ, D. S. AND AXELROD, J.: Delta-9-tetrahydrocannabinol: Localization in body fat. Science (Washington D.C.) **179:** 391–393, 1973.

LOPEZ-CEPERO, M., FRIEDMAN, M., KLEIN, T. W. AND FRIEDMAN, H.: Tetrahydrocannabinol-induced suppression of macrophage spreading and phagocytic activity *in vitro*. J. Leukocyte Biol. **39:** 679–686, 1986.

LYMAN, W. D., SONETT, J. R., BROSNAN, C. F., ELKIN, R. AND BORNSTEIN, M. B.: Δ^9-Tetrahydrocannabinol: A novel treatment for experimental autoimmune encephalomyelitis. J. Neuroimmunol. **23:** 73–81, 1989.

MARTIN, B. R.: Cellular effects of cannabinoids. Pharmacol. Rev. **38:** 45–74, 1986.

MATSUDA, L. A., LOLAIT, S. J., BROWNSTEIN, M. J., YOUNG, A. C. AND BONNER, T. I.: Structure of a cannabinoid receptor and functional expression of the cloned cDNA. Nature (Lond.) **346:** 561–564, 1990.

MECHOULAM, R., SHANI, A., EDERLY, H. AND GRUNFELD, Y.: Chemical basis of hashish activity. Science (Washington D.C.) **169:** 611–612, 1970.

MERRITT, J. C.: Glaucoma, hypertension and marijuana. J. Natl. Med. Assoc. **74:** 715–716, 1982.

MUNRO, S., THOMAS, K. L. AND ABU-SHAAR, M.: Molecular characterization of a peripheral receptor for cannabinoids. Nature (Lond.) **365:** 61–65, 1993.

MURPHY, L., DE FONSECA, F. R. AND STEGER, R. W.: Δ^9-Tetrahydrocannabinol antagonism of the anterior pituitary response to estradiol in immature female rats. Steroids **56:** 97–102, 1991.

NAHAS, G. G., SUCIA-FOCA, N., ARMAND, S. P. AND MORISHIMA, A.: Inhibition of cellular mediated immunity in marijuana smokers. Science (Washington D.C.) **183:** 419–420, 1974.

NOGAWA, T., SHINOHARA, O., HENRICH, R. AND MORISHIMA, A.: The effects of chronic administration of Δ^9-tetrahydrocannabinol on the early embryonic development of mice. *In* The Cannabinoids: Chemical, Pharmacological, and Therapeutic Aspects, ed. by S. Agurell, W. L. Dewey and R. E. Willette, pp. 345–357, Academic Press, New York, 1984.

NYE, J. S., SELTZMAN, H. H., PITT, G. C. AND SNYDER, S. H.: High affinity cannabinoid binding sites in brain membranes labelled with [^{3}H]-5′-trimethylammonium Δ^8-tetrahydrocannabinol. J. Pharmacol. Exp. Ther. **234:** 784–791, 1985.

NYE, J. S., VOGLMAIER, S., MARTENSON, R. E. AND SNYDER, S. H.: Myelin basic protein is an endogenous inhibitor of the high-affinity cannabinoid binding site in brain. J. Neurochem. **50:** 1170–1178, 1988.

OTTAWAY, C. A., LEWIS, D. L. AND ASA, S. L.: Vasoactive intestinal peptide-containing nerves in the Peyer's patches. Brain Behav. Immun. **1:** 148–158, 1987.

PATRA, P. B. AND WADSWORTH, R. M.: Effect of the synthetic cannabinoid nabilone on spermatogenesis in mice. Experientia **46:** 852–854, 1990.

PILLAI, R., NAIR, B. S. AND WATSON, R. R.: AIDS, drugs of abuse and the immune system: A complex immunotoxicological network. Arch. Toxicol. **65:** 609–617, 1991.

PROSS, S. H., KLEIN, T. W., NEWTON, C. A., SMITH, J., WIDEN, R. AND FRIEDMAN, H.: Differential suppression of T-cell subpopulations by THC (delta-9-tetrahydrocannabinol). Int. J. Immunopharmacol. **12:** 539–544, 1990.

ROSELL, S. AND AGURELL, S.: Effects of 7-hydroxy-Δ^6-tetrahydrocannabinol and some related cannabinoids on the guinea pig isolated ileum. Acta Physiol. Scand. **94:** 142–144, 1975.

RYRFELDT, Å. AND RAMSAY, C. H.: Whole-body autoradiography of Δ^1-tetrahydrocannabinol and $\Delta^{1(6)}$-tetrahydrocannabinol in mouse. Acta Pharm Suec. **10:** 13–28, 1973.

SHERMAN, M. P., CAMPBELL, L. A., GONG, H. J., ROTH, M. D. AND TASHKIN, D. P.: Antimicrobial and respiratory burst characteristics of pulmonary alveolar macrophages recovered from smokers of marijuana alone, smokers of tobacco alone, smokers of marijuana and tobacco, and nonsmokers. Am. Rev. Respir. Dis. **144:** 1351–1356, 1991.

SIGLINGA, N. AND GOLDSTEIN, A.: Opioid peptides and opioid receptors in cells of the immune system. Annu. Rev. Immunol. **6:** 219–249, 1988.

SPECTER, S., LANCZ, G. AND GOODFELLOW, D.: Suppression of human macrophage function *in vitro* by Δ^9-tetrahydrocannabinol. J. Leukocyte Biol. **50:** 423–426, 1991.

SPECTER, S., LANCZ, G., WESTRICH, G. AND FRIEDMAN, H.: Delta-9-tetrahydrocannabinol augments murine retroviral induced immunosuppression and infection. Int. J. Immunopharmacol. **13:** 411–417, 1991.

STANISZ, A. M., SCICCHITANO, R., DAZIN, P., BIENENSTOCK, J. AND PAYAN, D. G.: Distribution of substance P receptors on murine spleen and Peyer's patch T and B cells. J. Immunol. **139:** 749–754, 1987.

WHITE, S. C., BRIN, S. S. AND JANICKIWI, B. W.: Mitogen-induced blastogenic response of lymphocytes from marijuana smokers. Science (Washington D.C.) **188:** 71–72, 1975.

WIEDERMANN, C., SERTL, K. AND PERT, C.: Substance P receptors in rat spleen: Characterization and autoradiographic distribution. Blood **68:** 1398–1401, 1986.

Send reprint requests to: Miles Herkenham, Ph.D., Section on Functional Neuroanatomy, NIMH, Bldg. 36, Rm. 2D-15, Bethesda, MD 20892.