A similar oligosaccharide elution pattern was observed in urine samples from human patients affected with infantile or juvenile G_{M1} (*11*). In contrast the pattern of oligosaccharides extracted from the liver of dog 19 (Fig. 3B) was different from the pattern found in a dog with juvenile G_{M1} (Fig. 3C) (*12*). The latter lacked the major peak retention index 3.5 corresponding to an oligosaccharide with nine sugar residues.

Several different tissues from dog 26 and dog 28 were examined by HPLC for the presence of oligosaccharides. Oligosaccharides from samples of the spinal cord, vertebral disk, and cerebellum were characterized by a chromatographic pattern similar to that seen in the sample from the proband's liver (compare Fig. 3, B, D, and F). The chromatographic pattern obtained from dog 28, which was asymptomatic (Fig. 3E), was different, lacking the characteristic triplet of peaks with retention indices between 1.5 and 3.5 seen in the other extracts and in the urine.

These observations suggest that some English springer spaniel mutants are affected by a genetically determined lysosomal storage disease that closely resembles human infantile G_{M1}-gangliosidosis. These mutant dogs have neurovisceral and skeletal involvement, and an accumulation in affected organs of undegraded oligosaccharides that differ in their HPLC patterns from those reported for the canine model of juvenile G_{M1}-gangliosidosis (*12*). Analysis of the dogs' pedigree and clinical manifestations suggested transmission of the disease through an autosomal recessive pattern of inheritance with variable expressivity. This new canine mutant with β-galactosidase deficiency and multiple organ involvement is of particular interest because it provides a model to study human infantile G_{M1}-gangliosidosis and Morquio's disease type B. Furthermore, this animal model provides a versatile in vivo system for testing various therapeutic modalities such as enzyme replacement (*13*), bone marrow replacement (*14*), and gene insertion.

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Trans-4-Hydroxy-2-Hexenal: A Reactive Metabolite from the Macrocyclic Pyrrolizidine Alkaloid Senecionine

Abstract. *The toxicity of macrocyclic pyrrolizidine alkaloids in the livers of man and animals has been attributed to the formation of reactive pyrroles from dihydropyrrolizines. Now a novel metabolite,* trans*-4-hydroxy-2-hexenal, has been isolated from the macrocyclic pyrrolizidine alkaloid senecionine, in an in vitro hepatic microsomal system. Other alkenals such as* trans*-4-hydroxy-2-nonenal have previously been isolated from microsomal systems when treated with halogenated hydrocarbons or subjected to lipid peroxidation. The in vivo pathology caused by* trans*-4-hydroxy-2-hexenal appears to be identical to that previously attributed to reactive pyrroles. There are similarities between the toxic effects of this alkenal and those of centrilobular hepatotoxins such as CCl_4 and other alkenals formed during lipid peroxidation.*

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Plants containing pyrrolizidine alkaloids (PA's) are found throughout the world (*1*). These naturally occurring compounds are responsible for livestock losses and human poisonings (*2*). Many PA's are hepatotoxins and may cause an irreversible hemorrhagic necrosis, hepatic fibrosis, and megalocytosis (*3*). Some may also contribute to lung injury (*4*), and several have been shown to be teratogenic (*5*), carcinogenic (*6*), and genotoxic (*7*). Grains, bread, milk, honey, and herbal teas contaminated with PA's have either caused human poisonings or represent potential sources of human poisonings (*8*).

The major metabolites from unsaturated PA's, *N*-oxides, and pyrroles are generated by the action of the hepatic microsomal enzyme system (*9*). The pyrroles have been associated with the toxic effects of the PA's while the *N*-oxides are believed to result from a detoxification step in pyrrole metabolism (*10*). The pyrrole may be metabolized further (*11*), but efforts to isolate the final product have failed as the pyrrole derivative (or derivatives) is a reactive substance and rapidly decomposes or polymerizes in an aqueous environment (*12*).

It is possible that numerous metabolites and intermediates are responsible for syndromes associated with PA poisoning. The pyrrolizidine metabolite dehydroretronecine may covalently bind to cysteine, glutathione plus other thiol-containing compounds, as well as purines and pyrimidines such as adenosine monophosphate and deoxyguanosine (*12, 13*). By means of an in vitro mouse hepatic system and the macrocyclic PA senecionine derived from *Senecio vulgaris* (common groundsel), we have demonstrated that senecic acid, senecionine-*N*-oxide, 19-hydroxysenecionine, methoxydehydroretronecine, and hydroxydanaidal are formed from senecionine (*14*) (Fig. 1).

A [^{14}C] senecionine-derived metabolite has been difficult to identify; upon isolation it either decomposes, polymerizes, or forms a dark reddish-brown or purple precipitate after the addition of

deuterated chloroform ($CDCl_3$) (*15*). To verify that this ^{14}C-metabolite was derived from the retronecine portion of senecionine and not the senecic acid portion we used [^{14}C]senecionine that had been isolated from *S. vulgaris* plants treated with [2-^{14}C]ornithine (*7*). Upon hydrolysis, more than 95 percent of the radioactivity was incorporated into the retronecine portion (Fig. 1a, carbons 7, 8, and 9) of the [2-^{14}C]ornithine-derived senecionine (*16*). The remaining incorporated radioactivity was divided between the senecic acid and the unreacted senecionine.

[^{14}C]Senecionine derived from [2-^{14}C]ornithine or $^{14}CO_2$ and nonlabeled senecionine were used in separate in vitro mouse hepatic microsomal experiments (*14*). The reactions were stopped, and then the products were chromatographed with preparative and analytical reversed-phase chromatography (*17*). The labeled metabolite with 2.7 to 4.3 percent of the soluble radioactivity and a retention time of 33 minutes was derived from the retronecine base. Combined gas chromatography–mass spectrometry (GC-MS) of the isolated metabolite gave a molecular weight of 114 and tentative elemental composition $C_6H_{10}O_2$ (*18*)

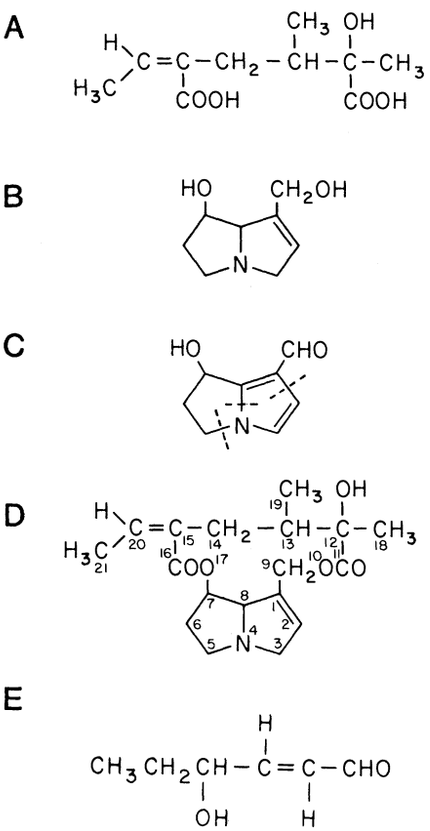

Fig. 1. Structure of (A) senecic acid, (B) retronecine, (C) hydroxydanaidal (dashes indicate possible formation of *t*-4HH), (D) senecionine, and (E) *t*-4HH.

(Fig. 2). The proton nuclear magnetic resonance (NMR) spectra indicated that the elusive metabolite was *trans*-4-hydroxy-2-hexenal (*t*-4HH) (*19*). This metabolite was synthesized and the identity confirmed by chromatography, NMR, and mass spectrometry (*18–20*). It appears that *t*-4HH is formed by further metabolism of the retronecine base arising from cleavage of bonds between C-1 and C-2, N and C-8, and C-5 and N (Fig. 1, B, C, and D).

Intraperitoneal injection of *t*-4HH in saline resulted in a severe localized necrotizing peritonitis, but no hepatic lesions. Injection of *t*-4HH in saline into the portal veins of rats created hepatic lesions similar to those reported for intravenous injection of dehydroretronecine (*12*). These lesions included thrombosis of larger intrahepatic veins and coagulative hepatocellular necrosis of parenchyma adjacent to these veins, frequently in a wedge-shaped region extending from one portal triad to the central vein.

To prevent the intravascular coagulation that occurred when *t*-4HH was administered in saline, we injected *t*-4HH or the parent compound (senecionine) into the portal vein as a suspension in a carrier lipid (*21*). Rats receiving *t*-4HH in this manner had large lesions in the left portion of the median lobe (*22*). These lesions covered one-third to one-half of the lobe and, in some animals, the medial portion of the right half of the median lobe or the hilar portion of the caudate lobe.

In animals treated with senecionine or *t*-4HH, affected areas of the liver had coalescing regions of coagulative hepatocellular necrosis that were focally bordered by a thin zone of degenerating neutrophils (Fig. 3). Senecionine-injected animals had lesions that were generalized but limited to centrilobular (zone 3) hepatocytes, while *t*-4HH lesions involved smaller areas of parenchyma but a greater proportion of the hepatic acinus in affected regions. In animals injected with *t*-4HH, islands of relatively unaffected hepatocytes frequently surrounded portal triads within regions of coagulative necrosis (Fig. 3). In other areas, small wedge-shaped foci of hepatocellular necrosis extended from zone 2 (midzonal) of individual acini to one side of the terminal hepatic vein. The regions of necrosis were most frequent in the peripheral portions of the median lobe and were also more frequent near the capsular surface (*23*). The livers of rats receiving only carrier lipid (controls) did not have any lesions visible to the unaided eye or microscopically.

The presence of the lesions in the median lobes of animals treated with senecionine or *t*-4HH probably reflects differential blood flow to these regions from the portal vein injection site. Necrosis of zone 3 hepatocytes is an established acute response to PA administration (*2*). Chemically induced zone 3 necrosis is thought to be a result of microvascular changes in the affected acini or metabolic heterogeneity of the hepatic acinus (or both) (*24*). Observed histologic patterns suggest that there was selective zone 3 necrosis of some acini while adjacent acini were unaffected (*25*). These lesions probably reflect differences in the concentrations of compound reaching different complex acini as a consequence of the intraportal injection (*23*).

Investigators studying the peroxida-

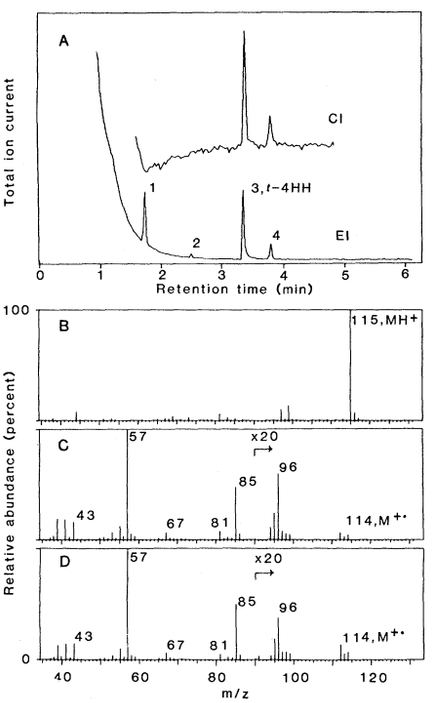

Fig. 2. GC-MS analysis of *t*-4HH. (A) Lower curve, total ion current (TIC) for GC-MS with electron ionization (EI). Peak 3, eluting at 3.2 minutes (column temperature, 87°C) is *t*-4HH. Peaks 1, 2, and 4 are solvent impurities, identified by comparison with reference mass spectra as isopropylbenzene, phenol, and hexachloroethane. Upper curve, TIC for GC-MS with isobutane chemical ionization (CI). (B) Isobutane CI spectrum of isolated metabolite (*t*-4HH). The protonated molecular ion (MH^+) at mass-to-charge ratio (*m/z*) 115 indicated a molecular weight of 114 for the metabolite, and the intensity ratio of *m/z* (116:115) of 7.0 percent suggested an elemental composition $C_6H_{11}O_2$ for MH^+ (calculated peak ratio based on ^{13}C natural abundance is 6.72 percent). (C) EI spectrum of isolated metabolite (*t*-4HH); *m/z* 114, molecular radical ion ($M^{+\cdot}$). (D) EI spectrum of synthetic *t*-4HH from GC-MS analysis under identical experimental conditions as in (C).

tion of hepatic microsomal lipids due to CCl_4 or $BrCCl_3$ toxicity have isolated aldehydes such as the 4-hydroxyalkenals, with the largest fraction being 4-hydroxynonenal (*26*). In addition to 4-hydroxynonenal, the compounds 4-hydroxyoctenal, 4-hydroxydecenal, 4-hydroxyundecenal, and 4,5-dihydroxydecenal have been isolated during the peroxidation of hepatic microsomal lipids induced by the complex of iron and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (*27*). These alkenal compounds have the ability to damage cellular sites distant from the initial site of peroxidation (*28*). The alkenals selectively inhibited microsomal enzymes (in vitro) such as aminopyrine demethylase, cytochrome P-450, and glucose-6-phosphatase but not NADPH–cytochrome *c* reductase. These results are in agreement with hepatic CCl_4 inhibition of membrane-bound enzymes and membrane damage in vivo (*28*). In addition, alkenals can lyse erythrocytes, inhibit protein synthesis, strongly inhibit adenyl cyclase, and kill isolated hepatocytes, which also correlates with in vivo hepatic CCl_4 inhibition (*28, 29*).

The 4-hydroxyalkenals are highly reactive electrophilic agents that react with the SH groups of low molecular weight thiols (glutathione, cysteine) as well as proteins by 1,4 addition to form Michael adducts (*30*). Initially, saturated aldehydes are formed by the addition of the SH group followed by the reaction of the free aldehyde with the hydroxyl group to yield a cyclic hemiacetal (*30*). This may be the reason that toxicity of PA's in mice and rats is decreased after glutathione or cysteine levels are raised (*31*).

Initial results in our laboratory indicate that aldehyde dehydrogenase may play an important role in detoxifying PA's. Since aldehyde dehydrogenase detoxifies aldehydes by converting them to acids, the isolation of the aldehyde *t*-4HH as a metabolite in PA metabolism may explain the protective role of aldehyde dehydrogenase in PA toxicity. Phenobarbital stimulates the Φ enzyme of aldehyde dehydrogenase, but not the τ enzyme of aldehyde dehydrogenase in rats (*32*), which may explain the mixed toxicity data obtained from pretreating animals with phenobarbital and then challenging them with PA's (*9, 33*). The cytosolic aldehyde dehydrogenase levels in pregnant mice increased threefold relative to nonpregnant mice (*34*). Peterson and Jago have shown that pregnant rats appear to have increased resistance to PA's (*5*).

In previous investigations of PA metabolism, *t*-4HH has not been considered to be a potential toxic metabolite, as the accepted theory stated that a pyrrole may be the final metabolite. Numerous pyrroles have been isolated in vitro (such as dehydroheliotridine, methoxydehydroretronecine, and hydroxydanaidal) as well as in vivo (dehydroretronecine) (*14, 35*). Further investigations into the detoxification of PA's by SH-containing compounds (such as glutathione and cysteine) as well as the inhibitory effects of aldehyde dehydrogenase on PA toxicity must be undertaken. The observations that glutathione and cysteine exerted a protective effect relative to PA toxicity can be explained by their interaction with *t*-4HH.

The identification of *t*-4HH as a metabolite that may exert its toxicity in a distant location may be important in elucidating the toxic nature of the PA monocrotaline on the heart and lung (*36*). The isolation of another 4-hydroxyalkenal (*t*-4HH) from the macrocyclic PA senecionine indicates that these aldehydes may be a more common factor in lipid peroxidation and hepatotoxicity than previously believed.

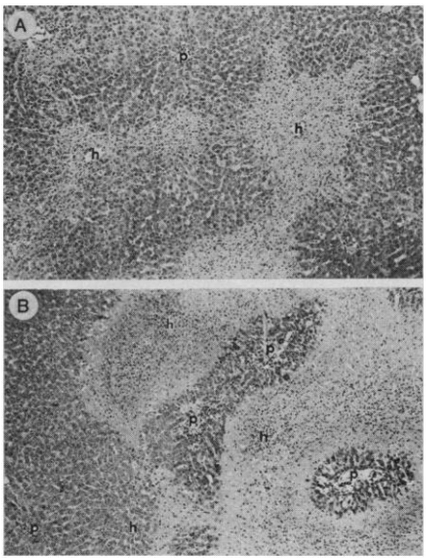

Fig. 3. (A) Rat liver stained with hematoxylin and eosin from a rat injected intraportally with senecionine in carrier lipid. Regions of coagulative necrosis in zone 3 of the hepatic acinus connect terminal hepatic veins (h) while zones 1 and 2 surrounding terminal portal veins (p) are relatively unaffected. Final magnification, ×67. (B) Photomicrograph of rat liver stained with hematoxylin and eosin from a rat injected intraportally with *t*-4HH in carrier lipid. Regions of coagulative necrosis in zone 3 and, to a lesser extent, zone 2 connect terminal hepatic veins (h), while zone 1 surrounding terminal portal veins (p) is relatively unaffected. An adjacent acinus (lower left) is also unaffected. Final magnification, ×67.

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15. Chromatography conditions were as described (*14*) with solvent system i. Peak II (*14*) had a retention time of 33 minutes. $CDCl_3$ contains a small amount of DCl which reacted with the aldehyde; personal communication, Technical Department, Aldrich Chemical Co.
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17. Incubation times and chromatography conditions were as described (*14*). A preparative system with solvent system ii and a semipreparative system with solvent system iv were used.
18. GC-MS analysis was done with a 15 m long 0.32-mm internal diameter fused silica glass capillary column (Quadrex Corp., New Haven, Conn.) coated to 0.25-μm film thickness with methyl silicone (stationary phase). The system was programmed to operate from 60°C to 200°C at 8°C per minute and directly coupled to an MM-70/70 HS mass spectrometer (VG Analytical, Ltd, Manchester, England) with injecter, transfer lines, and ion source temperatures all below 180°C. EI spectra were obtained at 70 electron volts. For isobutane chemical ionization, the source pressure was 3×10^4 pascals with the intensity ratio for *m/z* (57:43) of 1.8. The *t*-4HH eluted at 3.2 minutes with helium carrier gas at a linear velocity of 80 cm/sec.
19. The proton structure of *t*-4HH [O = CH^aCH^b = $CH^cCH^d(OH)CH_2{}^eCH_3{}^f$] shows absorption at 0.99 (triplet, 3*H*, H^f), 1.51 (multiplet, 2*H*, H^e), 3.47 (singlet, 1*H*, O*H*), 4.37 (multiplet, 1*H*, H^d), 6.30 (doublet of doublets, 1*H*, H^b), 6.79 (doublet of doublets, 1*H*, H^c), and 9.58 ppm (doublet, 1*H*, H^a), downfield from internal tetramethyl silane = 0 ppm (residual $CHCl_3$ = 7.24 in chlo-

roform d). The coupling constant J (in Hz) for each proton was $J_{a,b}$ (7.7), $J_{b,c}$ (15.7), $J_{b,d}$ (1.2), $J_{c,d}$ (4.7), $J_{e,f}$ (7.4). The value of $J_{b,c}$ (15.7 Hz) identifies a *trans* configuration between H^b and H^c; R. M. Silverstein, G. C. Bassler, T. C. Morrill, in *Spectrometric Identification of Organic Compounds* (Wiley, New York, 1974), p. 226. Spectra were recorded at ambient temperatures (23° to 25°C) with a Nicolet magnetic NMC500 spectrometer. Spectral resolution was <0.002 ppm. Resolution enhancement was achieved by double exponential apodization [A. G. Ferrige and J. C. Lindon, *J. Magn. Reson.* **31**, 337 (1978)]. Spectral ^1H assignments were achieved by nuclear spin decoupling, resonance integration, and comparison with previously published spectral assignments of PA's, dihydropyrrolizine alkaloids, and their metabolites [C. C. J. Culvenor, J. A. Edgar, L. W. Smith, H. J. Tweeddale, *Aust. J. Chem.* **23**, 1853 (1970); H. J. Segall and J. L. Dallas, *Phytochemistry* **22**, 1271 (1983)].
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21. The preparation of the lipid carrier and *t*-4HH or senecionine was as follows. Phosphatidylcholine was evenly coated on the sides of a test tube. Then, *t*-4HH was added in 1 to 2 ml phosphate-buffered saline (*p*H 7.4) with two glass beads. When senecionine was used, it was dissolved in 0.1*N* HCl before addition of buffer. The solution was vortexed for 1 minute followed by incubation for 10 minutes on ice; this procedure was repeated five times. Prior to injection, the solution was warmed to 37°C. A 10- to 12-mm incision was made on the ventral surface of the midline of male rats (110 to 125 g) and solutions were injected directly into the portal vein (*t*-4HH, 13 to 18 mg/kg; senecionine, 26 mg/kg).
22. Standard nomenclature for gross anatomy of the rat liver was used [E. C. Greene, in *Anatomy of the Rat* (Hafner, New York, 1963), p. 101; D. L. Knook and C. F. Hollander, in *Rat Hepatic Neoplasia*, P. M. Newberne and W. H. Butler, Eds. (MIT Press, Cambridge, 1978), pp. 8–42].
23. The administration of *t*-4HH as an intravenous bolus may have resulted in a greater proportion of the injected material entering one of the intrahepatic portal vein radicles. This would be due to differential blood flow rates or to the anatomic configuration of these vessels. As neighboring acini are supplied by different portal vein radicles [D. L. J. Bilbey and A. M. Rappaport, *Anat. Rec.* **136**, 165 (1960)], differing flow rates or proportion of dose to different radicles could account for the selective necrosis of some complex acini.
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Protein-Specific Helper T-Lymphocyte Formation Initiated by Dendritic Cells

Abstract. *Antibody responses to hapten-polypeptide conjugates require peptide-specific helper T cells. The latter can be primed in tissue culture by providing small numbers of dendritic cells. Primed, irradiated helper T cells then induce B-cell growth and differentiation in the apparent absence of dendritic cells. Both stages of the antibody response—the induction of helper T lymphoblasts by dendritic cells and the delivery of help from T to B cell—occur in discrete cell aggregates that can be isolated by velocity sedimentation. If helper T blasts revert to smaller "memory" lymphocytes, dendritic cells again are needed to initiate the antibody response.*

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Helper T lymphocytes enhance antibody responses to many antigens, such as foreign red blood cells and defined haptens coupled to polypeptide carriers (*1*). Carrier-specific helper cells usually are obtained from animals that have been primed by exposure to antigen in vivo. This approach, however, makes it difficult to control the determinants that are to be recognized and to analyze the cellular requirements for the induction of helper cells. These topics are assuming increasing importance because of the interest in understanding the initiation of immune responses and the use of polypeptide vaccines. We report here the successful priming of polypeptide-specific helper T-lymphocytes in vitro.

The experimental approach required the use of dendritic cells (DC's) as accessory cells (*2*). DC's are irregularly shaped leukocytes first identified in the adherent population of mouse spleen cells (*3*). At that time it was known that cells adherent to plastic or glass enhanced antibody formation by nonadherent B and T lymphocytes (*4*). Once DC's were separated from other adherent cells, particularly macrophages (*5*), it became apparent that DC's were specialized stimulators of many immune responses, including antibody formation to T cell–dependent antigens (*2, 6*). We will show here that a special role of DC's is to generate active helper cells from unprimed and memory T lymphocytes.

Spleen cell suspensions (from CXD2 F1 mice unless otherwise indicated) were passed over Sephadex G10 columns to provide mixtures of B and T lymphocytes. The latter mixtures were effectively depleted of DC's, as evidenced by their inability to stimulate mixed-leukocyte reactions (MLR's) and to form antibodies (*2*). B cells were G10-nonadherent cells that were treated with antibodies to Thy-1 and Lyt-1 and complement to eliminate T cells. Helper T cells were nylon wool–nonadherent spleen and lymph node cells that were treated with antibodies to I region–associated antigen (Ia) and to Lyt 2.2 and with complement to eliminate residual B cells, DC's, and cytotoxic and suppressor cells. Macrophages were firmly adherent peritoneal or spleen cells. DC's were plastic-adherent spleen cells that had been depleted of macrophages after overnight culture. Although DC's and macrophages are both derived from an initial adherent population, DC's are nonphagocytic, become nonadherent after overnight culture, express the 33D1 DC-specific marker, and lack Fc receptors (*5*). The efficacy of these enrichment procedures was verified by sensitive assays. The B cells responded to the B-cell mitogen LPS (33,000 count/min per 10^5 cells) but not to the T-cell mitogen concanavalin A (Con A) (300 count/min per 10^5 cells) nor to the fetal calf serum that was used in our cultures. The T cells did not respond to LPS (300 count/min per 10^5 cells) but did respond to Con A if small numbers of DC's were present (282,000 count/min per 10^5 T cells and 10^4 DC's). The DC's did not respond to either mitogen.

The relative efficacy of DC's and mac-