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An assessment of the acute effects of the serotonin releasers methylenedioxymethamphetamine, methylenedioxyamphetamine and fenfluramine on immunity in rats

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Abstract

The purpose of the present study was to examine the effect of the serotonin releasing amphetamine derivatives methylenedioxymethamphetamine (MDMA), methylenedioxyamphetamine (MDA) and fenfluramine (FEN) on immunity in rats. Similar to MDA and MDMA, FEN reduced the number of circulating lymphocytes, provoked a suppression of Con A-stimulated lymphocyte proliferation and total IFN-γ and IL-10 production in diluted whole blood cultures. Thus the non-psychostimulant amphetamine derivative FEN, shares the ability of the psychostimulant methylenedioxy-substituted amphetamine derivatives to alter these indices of immune function in the rat. However, when Con A-stimulated cytokine production was normalised for the number of lymphocytes in culture in order to examine cytokine production at a cellular level, the effect of the amphetamine derivatives begins to diverge. FEN shares with MDMA and MDA the ability to suppress production of the Th2 type cytokine IL-10. However the effect of these drugs on Th1 type cytokine secretion was much more complex. While the methylendioxy-substituted amphetamines increases the secretion of the Th1 type cytokine IL-2 without altering the related Th1 type cytokine IFN-γ, FEN did not alter IL-2 secretion, but suppressed IFN-γ secretion. In addition to these effects on T-cell responses, all three drugs inhibited LPS-induced TNF-α secretion from diluted whole blood cultures suggesting that macrophage activity is impaired following treatment. In all, these data extend our previous findings concerning the effects of MDMA on the immune system and demonstrate that the related serotonin releasers MDA and FEN also provoke immunological changes in rats. © 2000

Keywords: Cytokine; Fenfluramine; Immune system; IFN-γ; IL-1β; IL-2; IL-10; Immunosuppression; Lymphocytes; MDA; MDMA; Serotonin releasers; TNF-α

1. Introduction

3,4-Methylenedioxymethamphetamine (MDMA; ''Ecstasy'') is a ring substituted phenylisopropylamine that is structurally related to both amphetamines and hallucinogens. The unique behavioural activating properties of MDMA have led to its widespread abuse in humans. In laboratory animals, acute MDMA administration causes an increase in the release of serotonin, increased locomotor activity, changes in body temperature and activation of both the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system (SNS)

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(McNamara et al., 1995a,b; Stone et al., 1986; Grob et al., 1996; Gudelsky and Nash, 1996). In addition, we have recently reported that acute MDMA administration provokes a suppression of lymphocyte proliferation in response to the T-cell mitogens concanavalin A (Con A) and phytohemagglutinin (PHA) and reduced the number of circulating lymphocytes in rats (Connor et al., 1998, 1999a; Kelliher et al., 1998). This reduction in lymphocyte proliferation and circulating leucocyte numbers persisted for at least 6 h following acute MDMA administration in rats (Connor et al., 1998), indicating that MDMA abuse may have immunotoxic potential and thus lead to increased disease susceptibility. In addition to our studies, a recent report indicates the acute MDMA administration suppresses lymphocyte proliferation, but enhances NK-cell activity in mice, and that these immunological alterations are only partially restored 24 h following MDMA administration (Pacifici et al., 1999).

It is now known that two distinct subsets of helper T-cells exist, Th1 and Th2 (Mosmann et al., 1986), and these subsets are defined by their profile of cytokine production and resultant activation of different immune responses (Mosmann and Coffman, 1989). Th1 cells predominantly produce interleukin (IL)-2, interferon (IFN)-γ and tumor necrosis factor (TNF)-β and are generally associated with inducing cell mediated immune responses. On the other hand Th2 cells produce mainly IL-4, IL-5, IL-6 and IL-10, which are generally associated with promoting humoral immune responses. Cytokines are required for almost all immunoregulatory activities, and thus represent an important potential target for drug related immunomodulation. In this regard it was recently suggested that a promising new avenue for early detection of immunotoxicity may be the measurement of the expression or secretion of various cytokines (Pallardy, 1998). In addition, a World Health Organisation (WHO) directive has also suggested that changes in the pattern of cytokine expression may serve as an early indicator of immunotoxicity (WHO, 1996).

In the present study we examined the effect of MDMA on Th1 (IL-2 and IFN-γ) and Th2 (IL-10) type cytokine production in whole blood cultures and whole blood lymphocyte proliferative responses following stimulation with the T-cell mitogen Con A. LPS-stimulated IL-1β and TNF-α secretion were also measured in whole blood cultures as an index of macrophage function following MDMA administration. IL-1 is a proinflammatory cytokine released from activated macrophages. During inflammation, injury and immunological challenge or infection IL-1 is produced and because of its multiple biological properties this cytokine is of primary and strategic importance to the outcome of disease, particularly inflammatory and infectious disease (see Dinarello, 1994). TNF-α, like IL-1, is also a proinflammatory cytokine released from activated macrophages. TNF-α is a vital component of the cellular immune response (see Beutler, 1995) and is a key mediator of inflammation and the mammalian host's response to neoplasia, injury or invasion by bacteria, viruses and parasites (Wong and Goeddel, 1986; Tracey, 1994). In the present study a whole blood method was used for the assessment of both lymphocyte proliferation and cytokine production. In diluted whole blood the natural cell–cell interactions are preserved, whereas the methods used to isolate peripheral blood mononuclear cells (PBMCs) modify the lymphocyte/monocyte ratio and eliminate endogenous immunomodulatory agents. Thus in vivo conditions are better represented using whole blood culture methods (DeGroote et al., 1992; Zangerle et al., 1992), whilst the variability in cytokine production by isolated PBMC cultures is much larger than in diluted whole blood cultures (DeGroote et al., 1992). Also the diluted whole blood lymphocyte proliferation assay has been optimized for use with rat blood (Fasanmade and Jusko, 1995), and is routinely used for immunological assessments in rodents (Bayer et al., 1995; Mellon and Bayer, 1999; Pezzone et al., 1992).

In addition to MDMA, in the present study we also examined the immunological effects following the administration of two related substituted amphetamines namely methylenedioxyamphetamine (MDA) and fenfluramine (FEN). MDA is structurally related to MDMA and shares its serotonin releasing (Kankaanpaa et al., 1998; Wichems et al., 1995) and behaviourally stimulating properties (Hegadoren et al., 1995; Yeh and Hsu, 1991). In fact, following in vivo administration of MDMA, it is N-demethylated to produce MDA (Chu et al., 1996). Thus it is reasonable to suggest that in vivo

MDA may well be responsible for some of the effects observed following MDMA administration. However to date the effects of MDA have not been evaluated on the immune system. Like MDMA and MDA, FEN is also a potent releaser of central 5-HT (Wichems et al., 1995) but has a very different psychopharmacological profile to that of the methylenedioxy-substituted amphetamines. FEN does not have the abuse potential of MDMA and MDA as evidenced by extensive clinical experience, and lack of activity in animal models of drug self administration (Dahl and Gotesta, 1989; Pinder et al., 1975). To date, the few studies that investigated the effect of FEN on immunity have usually examined low subanorexic doses and have not verified that FEN provoked alterations in central serotonergic activity (Clancy et al., 1991; Clancy and Lorens, 1996; Mathews et al., 1996).

Thus in the present investigation we examined the effect of MDMA, MDA and FEN on total and differential leucocyte counts, Con A-stimulated lymphocyte proliferation and production of Th1 type (IL-2 and IFN-γ) and Th2 type (IL-10) cytokines and LPS-stimulated production of the macrophage derived cytokines IL-1β and TNF-α. We also compared the ability of the three compounds to increase locomotor activity in order to compare the behavioural effects of these amphetamine derivatives. In addition, cortical serotonin concentrations were measured in order to confirm the ability of these three drugs to alter central serotonergic activity. It was of particular interest to see if acute administration of the non-psychostimulant amphetamine FEN, would provoke immunological changes similar to that of MDMA and MDA in our rodent model, as FEN shares the ability of MDMA and MDA to activate the HPA-axis (McGarvey et al., 1995) and sympathetic nervous system (Arase et al., 1988). Activation of these two systems has been shown to mediate immunosuppressive effects of both drug treatments (Pezzone et al., 1992; Elenkov et al., 1995) and stressors (Cunnick et al., 1990; Fleshner et al., 1996; see Friedman and Irwin, 1997). In addition, the common neurochemical action of MDMA, MDA and FEN as potent releasers of the neurotransmitter serotonin, provide an ideal opportunity to examine the role of serotonin release in immunomodulation.

2. Materials and methods

2.1. Subjects

Female Sprague–Dawley rats weighing approximately 220–250 g were obtained from a Departmental breeding colony and housed in groups of four per cage. The rats were maintained on a 12 h:12 h light:dark cycle (lights on at 0800 h) in a temperature controlled room (22–24°C) and food and water were available ad libitum.

The experimental protocol was carried out under the guidelines of the Animal Welfare Committee, National University of Ireland, Galway, Ireland, and were in compliance with the European Communities council directive (86/609/EEC).

2.2. Drug administration

MDMA, MDA (Plaistow, Cork, Ireland) and fenfluramine (Sigma, Poole, Dorset, UK) were dissolved in 0.89% NaCl to give a concentrations of 10 mg/ml. 0.89% NaCl was administered alone as a vehicle to the control group. All drugs were administered in an injection volume of 1 ml/kg using the intraperitoneal (i.p.) route ($n = 8$ per group). A 10 mg/kg dose was chosen for two reasons. Firstly, because we have previously found that 7.5–10 mg/kg MDMA provokes a reduction in circulating leucocyte numbers and a suppression of lymphocyte proliferation in rats (Kelliher et al., 1998; Connor et al., 1999a). Secondly, we have previously demonstrated that administration of 10 mg/kg MDMA to rats yields serum concentrations of the drug which are within the range seen in human MDMA abusers (Connor et al., 2000). The other two drugs used in the present study MDA and FEN are not abused in humans per se, however we used the same dose of MDA and FEN as we did MDMA in order to compare the immunological effects of the three serotonin releasers. However, it should be noted that FEN has been used in the past as an anti-obesity drug and the doses used for this purpose would be lower than what we used in the present study, therefore it is noteworthy that doses of FEN as low as 2.5 mg/kg also suppress lymphocyte proliferation and reduce circulating WBC numbers in rats (unpublished data).

2.3. Homecage locomotor activity measurements

A separate group of singly housed rats ($n = 6$ per treatment group) were used to examine the effects of the amphetamine derivatives on locomotor activity. The locomotor activity of each animal was measured using an automated homecage activity monitor by means of an infrared sensor mounted above each cage. The sensors detected ambulation and were connected via an interface to an IBM personal computer (see McGarvey et al., 1995). On the day of the experiment monitoring commenced 30 min prior to drug administration in order to establish a baseline, and for 3 h following drug administration.

2.4. Determination of cortical serotonin concentrations

The rats were killed by decapitation while under ether anaesthesia 3 h after drug administration. After sacrifice, the brain was rapidly removed and the left frontal cortex was dissected on an ice cold plate (Popov et al., 1967). Serotonin concentrations were measured by high performance liquid chromatography (HPLC) coupled with electrochemical detection (Seyfried et al., 1986). The frontal cortex was homogenized by sonication in 1.0 ml of mobile phase (pH 2.8) that contained 2 ng/50 μl of N-methyl dopamine (Sigma, Poole, Dorset, UK) as an internal standard. The mobile phase contained 0.1 M citric acid (BDH Chemicals, Poole, Dorset, UK), 0.1 M sodium dihydrogen phosphate (BDH Chemicals, Poole, Dorset, UK), 1.4 mM octane-1-sulphonic acid (Sigma, Poole, Dorset, UK), 0.1 mM EDTA (BDH Chemicals, Poole, Dorset, UK) and 10% (v/v) methanol (Lab-Scan, Dublin, Ireland) and was adjusted to pH 2.8 using 4N NaOH (BDH Chemicals, Poole, Dorset, UK). Homogenates were centrifuged at 12,000 rpm in a Hettich Mikro/K refrigerated centrifuge for 15 min. A 20 μl sample of the supernatant was injected directly into a reverse phase column (LI Chrosorb RP-18, 25 cm × 4 mm internal diameter, particle size 5 μm) for separation of indoles and catecholamines (flow rate 1 ml/min). An electrochemical detector (Shimadzu) was coupled to the HPLC system and was set at a potential of +0.8 V for the detection of serotonin. Serotonin concentrations were quantified using a Merck-Hitachi D-2000 integrator and were expressed as ng of neurotransmitter per gram fresh weight of brain tissue.

2.5. Total and differential leucocyte counts

Prior to sacrifice the animals were anaesthetized with diethyl ether (Lab Scan, Dublin, Ireland) and a blood sample for the immune assays was obtained by cardiac puncture into a heparinized syringe. The total leucocyte counts were performed on heparinized whole blood samples using a haematology counter (Baker Diagnostics, Serono 9000). Blood smeared slides were stained with Wright's stain using an automated slide stainer (Ames, HEMA-TEK, Japan). The relative leucocyte percentages were counted on each slide using standard hospital procedures, under a microscope. The absolute numbers of lymphocytes, neutrophils and monocytes were calculated by multiplying the total leucocyte count by the relative percentages and dividing by one hundred.

2.6. Concanavalin A-stimulated whole blood lymphocyte proliferation

Whole blood lymphocyte proliferation was performed as previously described (Fasanmade and Jusko, 1995; Bayer et al., 1995; Pezzone et al., 1992). Heparinized blood was mixed with complete RPMI 1640 medium [RPMI 1640 + 10% (v/v) heat inactivated fetal calf serum +2% (v/v) penicillin/streptomycin] (Gibco Life Technologies, Scotland) (1:10 dilution). Briefly, 200 μl aliquots of diluted whole blood were pipetted in triplicate into wells of a sterile flat bottomed 96 well plate (Starstedt, Ireland). To each well was added either no mitogen for the unstimulated cultures or Con A (Sigma, Poole, Dorset, UK) at working concentrations of 2.5, 5.0 or 10 μg/ml. Cultures were incubated for 48 h at 37°C in a 5% CO_2 atmosphere prior to addition of [^{3}H]-thymidine (0.5 μCi/well). The cultures were then incubated for a further 24 h. At the end of the incubation period the plates were removed and stored at −70°C until they were harvested onto GF/C filters using a cell harvester (Brandel). [^{3}H]-thymidine uptake was measured using a liquid scintillation counter. Mean scintillation disintegrations per minute (DPM) were calculated for each concentration of mitogen. The lymphocyte proliferation data were

expressed as both total proliferation in whole blood cultures (DPM) (Fig. 1a) and proliferation which was normalized for the number of lymphocytes in culture (Fig. 1b) i.e., DPM per 10^6 lymphocytes/ml. Due to the fact that the drug treatments used in the present study significantly altered circulating lymphocyte numbers, it was necessary to normalize the proliferation data for lymphocyte number in order to assess the effect of drug treatments on lymphocyte proliferation at a cellular level.

2.7. Mitogen-stimulated whole blood cytokine production

Heparinized blood was mixed with complete RPMI 1640 medium (Gibco Life Technologies, Scot-

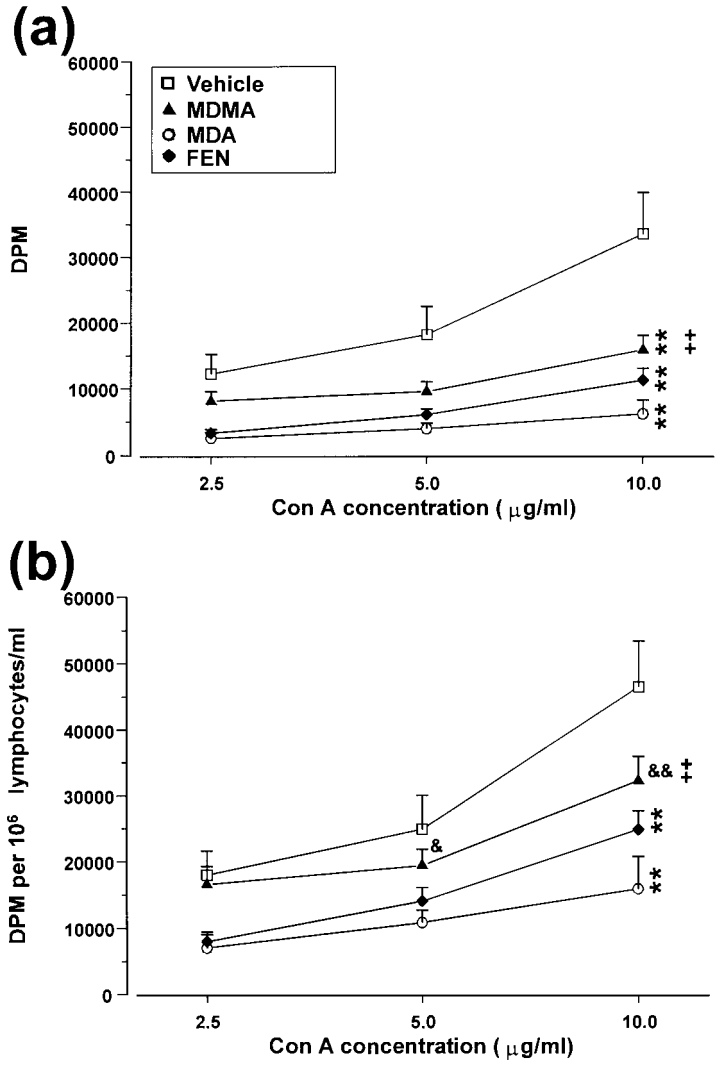

Fig. 1. Effect of acute MDMA, MDA and FEN administration on Con A-stimulated lymphocyte proliferation in diluted whole blood cultures (a) prior to and (b) following normalisation for the number of lymphocytes in culture. Data expressed as group means with standard errors. ($N = 8$) ** $P < 0.01$ vs. Vehicle across all doses of Con A, ++ $P < 0.01$ vs. MDA and FEN across all doses of Con A, & $P < 0.05$, && $P < 0.01$ vs. Vehicle (Fishers LSD).

land) (1:10 dilution) as previously described (DeGroote et al., 1992). Briefly, 1 ml aliquots of diluted whole blood were pipetted into wells of a sterile flat bottomed 24 well plate (Starstedt, Ireland). To each well was added either Con A (Sigma, Poole, Dorset, UK) at a working concentration of 5.0 μg/ml or lipopolysaccharide (LPS) (Sigma, Poole, Dorset, UK) at a working concentration of 25 μg/ml. Cultures were incubated for 72 h at 37°C in a 5% CO_2 atmosphere. At the end of the culture period the contents of each well was transferred into Eppendorf tubes and centrifuged at 12,000 RPM for 15 min at 4°C. Following centrifugation aliquots of the supernatant were stored at −70°C until cytokine assays were performed.

IL-2, IL-10 and IFN-γ were measured from Con A-stimulated culture supernatants, whilst IL-1β and TNF-α were measured on LPS-stimulated culture supernatants.

2.8. Cytokine assays

TNF-α and IFN-γ concentrations were measured in culture supernatants using commercially available rat ELISA kits (Genzyme, UK). IL-2 and IL-10 were also determined using commercially available rat ELISA kits (Biosource, UK). Results were expressed as pg/ml for total cytokine production in whole blood cultures (Table 2a). Due to the fact that the drug treatments used in the present study significantly altered circulating lymphocyte numbers, Con A-stimulated whole blood cytokine production was normalized for the number of lymphocytes in culture in order to assess the effect of drug treatments on cytokine production at a cellular level (Table 2b).

IL-1β concentrations were determined using a specific rat IL-1β ELISA sandwich assay performed using antibodies and standards obtained from Dr. S. Poole (NIBSC, Potters Bar, Herts, UK). Briefly, flat bottomed 96 well Maxisorp microtitre plates (Nunc, Belgium) were coated with immunoaffinity-purified sheep anti-rat IL-1β polyclonal antibodies (2 μg/ml in bicarbonate coating buffer; 0.1 M $NaHCO_3$, 0.1 M NaCl, pH 8.2, for 20 h at 4°C), then washed three times with wash/dilution buffer (0.5 M NaCl, 2.5 mM NaH_2PO_4, 7.5mM Na_2HPO_4, 0.1% Tween 20, pH 7.2). 100 μl of a 1% ovalbumin (Sigma, Poole, Dorset, UK) solution in bicarbonate coating buffer was added to each well and incubated at 37°C for 1 h. Following three washes 100 μl aliquots of samples and standards (recombinant rat IL-1β, 0–1000 pg/ml) were added and plates were incubated at 4°C for 20 h. After three washes 100 μl of the biotinylated sheep anti-rat IL-1β antibody (1:1000 dilution in wash buffer containing 1% sheep serum, Sigma, Poole, Dorset, UK) was added to each well. A further incubation was carried out for 1 h at room temperature. After three washes 100 μl avidin–HRP (Dako, UK) (1:5000 dilution in wash buffer) was added to each well and plates were incubated at room temperature for 15 min. Following three washes 100 μl of TMB substrate solution (Dako, UK) was added per well and the plates were incubated for 10 min at room temperature. At the end of the incubation period 100 μl of 1 M H_2SO_4 was added per well to stop the reaction and to facilitate colour development. Absorbance was read immediately at 450 nm on a microtitre plate reader (EL × 800, Bio-Tek instruments). Results were expressed as pg IL-1β/ml.

2.9. Statistical analysis of data

All data with the exception of the lymphocyte proliferation were analysed by a one-way analysis of

Table 1
Effect of acute administration of MDMA, MDA and FEN on circulating leucocyte numbers

Group	WBC ($\times 10^6$/ml)	Lymphocytes ($\times 10^6$/ml)	Neutrophils ($\times 10^6$/ml)	Monocytes ($\times 10^6$/ml)
Vehicle	7.8 ± 0.5	7.0 ± 0.5	0.7 ± 0.1	0.1 ± 0.04
MDMA	5.4 ± 0.5**	4.8 ± 0.5**	0.5 ± 0.1	0.1 ± 0.02
MDA	5.0 ± 0.7**	3.6 ± 0.3**	1.2 ± 0.4	0.1 ± 0.05
FEN	5.1 ± 0.4**	4.5 ± 0.4**	0.5 ± 0.8	0.1 ± 0.03

** $P < 0.01$ vs. Vehicle (Fishers LSD).

Table 2

(a) Effect of acute administration of MDMA, MDA and FEN on total Th1 (IL-2 and IFN-γ) and Th2 (IL-10) type cytokine production in Con A-stimulated diluted whole blood cultures

Group	IL-2 (pg/ml)	IFN-γ (pg/ml)	IL-10 (pg/ml)
Vehicle	139 ± 31	1486 ± 47	444 ± 83
MDMA	186 ± 17	1183 ± 118*	215 ± 35**
MDA	133 ± 19	680 ± 95**	114 ± 14**
FEN	106 ± 9	570 ± 125**	95 ± 16**

(b) Effect of acute administration of MDMA, MDA and FEN on Th1 (IL-2 and IFN-γ) and Th2 (IL-10) type cytokine production in Con A-stimulated diluted whole blood cultures which have been normalised for the number of lymphocytes in culture

Group	IL-2 (pg/10^6 lymphocytes)	IFN-γ (pg/10^6 lymphocytes)	IL-10 (pg/10^6 lymphocytes)
Vehicle	210 ± 50	2190 ± 130	630 ± 110
MDMA	420 ± 70**	2550 ± 310	440 ± 40*
MDA	380 ± 50*	1880 ± 210	320 ± 30**
FEN	250 ± 40	1210 ± 210*	210 ± 30**

*$P < 0.05$, **$P < 0.01$ vs. Vehicle (Fishers LSD).

variance. Lymphocyte proliferation data were analysed using a two-way analysis of variance with repeated measures for mitogen concentration. If any statistically significant change was found, post hoc comparisons were performed using Fisher's LSD multiple range test. Data were deemed significant when $P < 0.05$. Results are expressed as group mean with standard errors.

3. Results

3.1. Total and differential leucocyte counts

There was a significant effect of drug treatment on total leucocyte count [$F(3,28) = 5.37$, $P < 0.01$]. Post hoc analysis revealed a significant ($P < 0.01$) reduction in the number of circulating leucocytes after administration of MDMA, MDMA and FEN. There was also a significant effect of drug administration on the absolute lymphocyte count [$F(3,28) = 9.93$, $P < 0.0001$], post hoc analysis showing a significant ($P < 0.01$) reduction in circulating lymphocyte numbers following administration of all three amphetamine derivatives. Drug treatment failed to significantly alter absolute neutrophil or monocyte counts (Table 1)

3.2. Con A-stimulated whole blood lymphocyte proliferation

The ANOVA of Con A-stimulated lymphocyte proliferation demonstrated that there was a significant drug treatment × Con A concentration interaction [$F(6,56) = 18.72$, $P < 0.0001$]. Post hoc analysis showed that there was a significant reduction in Con A-induced lymphocyte proliferation following MDMA, MDA and FEN ($P < 0.01$) administration at all doses of mitogen used. In addition the effect of MDMA on Con A-induced lymphocyte proliferation was significantly less potent that of MDA and FEN ($P < 0.01$) at all doses of mitogen used (Fig. 1a). When the proliferation data was normalized for the number of lymphocytes in culture there was also a significant drug treatment × Con A concentration interaction [$F(6,56) = 6.17$, $P < 0.0001$]. In addition, post hoc analysis showed that there was a significant reduction in Con A-induced lymphocyte proliferation following MDA and FEN ($P < 0.01$) administration at all doses of mitogen. The effect of MDMA on Con A-induced lymphocyte proliferation was significantly less potent that of MDA and FEN ($P < 0.01$) at all doses of mitogen and was significantly reduced from the vehicle treated group at 5 ($P < 0.05$) and 10 ($P < 0.01$) μg/ml Con A (Fig. 1b).

3.3. Con A-stimulated Th1 and Th2 type cytokine production

There was no significant effect of drug administration on total IL-2 production in whole blood culture (Table 2a). However when the data was normalized for lymphocyte number there was a significant effect of drug treatment on Con A-stimulated IL-2 production [$F(3,28) = 3.83$, $P < 0.05$]. Post hoc analysis showed that there was a significant increase in IL-2 production proliferation after MDMA ($P < 0.01$) and MDA ($P < 0.05$) administration. FEN administration did not significantly alter IL-2 production (Table 2b).

There was a significant effect of drug treatment on Con A-stimulated total IFN-γ production [$F(3,28) = 18.16$, $P < 0.0001$]. Post hoc analysis showed that there was a significant decrease in IFN-γ production following MDMA ($P < 0.05$), MDA and FEN ($P < 0.01$) administration (Table 2a). When the data was normalized for lymphocyte number there was also a significant effect of drug treatment on Con A-stimulated IFN-γ production [$F(3,28) = 6.56$, $P < 0.01$]. However post hoc analysis showed that there was a significant increase in IFN-γ production following FEN administration ($P < 0.01$), whereas neither MDMA or MDA administration significantly altered IFN-γ secretion (Table 2b).

There was a significant effect of drug treatment on Con A-stimulated total IL-10 production [$F(3,28) = 11.82$, $P < 0.0001$]. Post hoc analysis showed that there was a significant decrease in IL-10 production following MDMA, MDA and FEN ($P < 0.01$) administration (Table 2a). When the data was normalized for lymphocyte number there was also a significant effect of drug treatment on Con A-stimulated IL-10 production [$F(3,28) = 8.33$, $P < 0.001$] and post hoc analysis showed that there was a significant decrease in IL-10 production following MDMA ($P < 0.05$), MDA and FEN ($P < 0.01$) administration (Table 2b).

3.4. LPS-stimulated IL-1β and TNF-α secretion

There was a significant effect of drug treatment on LPS-stimulated TNF-α production [$F(3,27) = 8.05$, $P < 0.001$]. Post hoc analysis showed that there was a significant decrease in TNF-α production following MDMA, MDA and FEN ($P < 0.01$) administration. In contrast LPS-stimulated IL-1β secretion was not significantly altered following drug administration (Fig. 2).

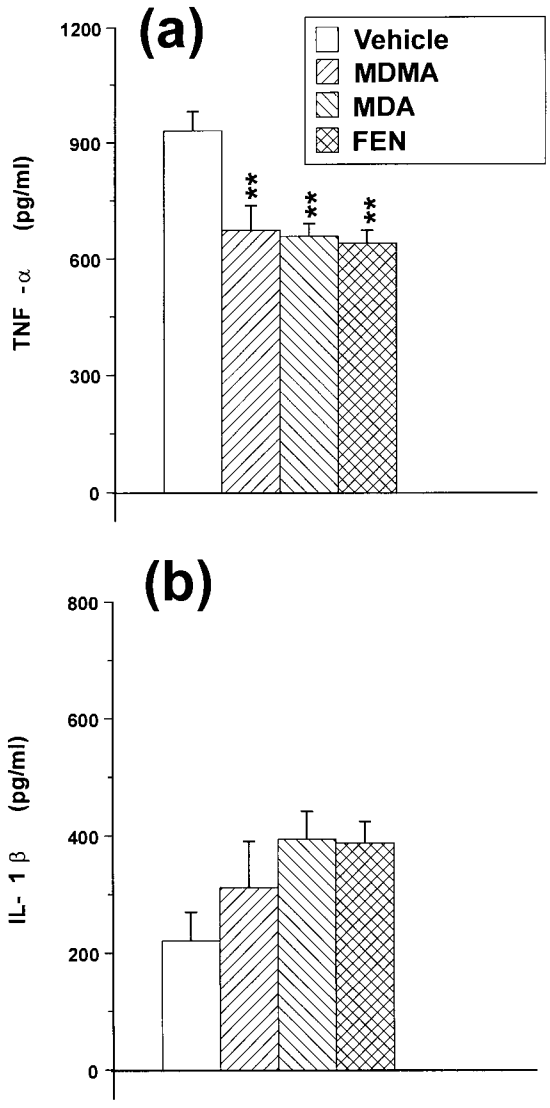

Fig. 2. Effect of acute MDMA, MDA and FEN administration on LPS-stimulated (a) TNF-α and (b) IL-1β production in diluted whole blood culture. Data expressed as group means with standard errors. ($N = 7–8$) ** $P < 0.01$ vs. Vehicle (Fisher's LSD).

3.5. Homecage locomotor activity

There was a significant effect of drug administration on homecage locomotor activity [$F(3,20) = 22.85$, $P < 0.0001$]. Post hoc analysis revealed a significant increase ($P < 0.01$) in homecage locomotor activity counts in the 3 h period following administration of MDMA and MDA. By contrast, FEN did not significantly alter locomotor activity (Fig. 3).

3.6. Cortical serotonin concentrations

There was a significant effect of drug administration on 5-HT [$F(3,23) = 9.71$, $P < 0.001$] in the frontal cortex. Post hoc analysis revealed a significant ($P < 0.01$) reduction in cortical 5-HT concentrations in response to all three drugs. There was no significant effect of drug treatment on 5-HIAA concentrations in the frontal cortex. When the 5-HIAA/5-HT ratio was used as an index of 5-HT turnover, there was a significant effect of drug treatment [$F(3,28) = 3.37$, $P < 0.05$]. Post hoc analysis revealed a significant increase ($P < 0.05$) in the cortical 5-HIAA/5-HT ratio following all three drug treatments (Table 3).

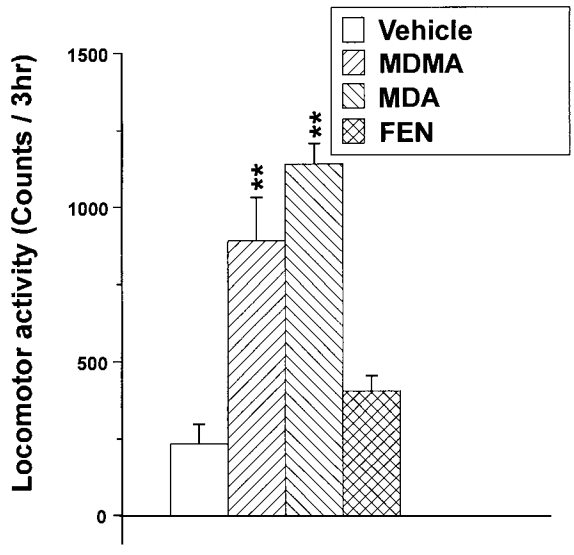

Fig. 3. Effect of acute MDMA, MDA and FEN administration on homecage locomotor activity. Data expressed as group means with standard errors. ($N = 6$) ** $P < 0.01$ vs. Vehicle (Fisher's LSD).

Table 3
Effect of acute administration of MDMA, MDA and FEN on serotonergic measures in the frontal cortex

Group	5-HT	5-HIAA	5-HIAA/5-HT
Vehicle	782 ± 75	504 ± 67	0.76 ± 0.16
MDMA	324 ± 48**	414 ± 37	1.40 ± 0.15*
MDA	341 ± 90**	356 ± 48	1.37 ± 0.22*
FEN	287 ± 37**	466 ± 55	1.38 ± 0.13*

* $P < 0.05$, ** $P < 0.01$ vs. Vehicle (Fishers LSD).

4. Discussion

In the present study all three amphetamine derivatives provoked a profound (32–49%) reduction in the total blood leucocyte count, which could be attributed to a net decrease in absolute lymphocyte numbers without any significant alteration in the circulating numbers of neutrophils or monocytes. These effects are similar to those previously observed with MDMA in our laboratory (Connor et al., 1998, 1999a,b; Kelliher et al., 1998). All three drugs also provoked a suppression of the lymphocyte proliferative response to the T-cell mitogen Con A, with MDA and FEN being more potent than MDMA in this regard. It is also of interest that the parent compound amphetamine also provokes a suppression of Con A-stimulated T-cell proliferation in rats (Pezzone et al., 1992). The suppression of Con A-stimulated lymphocyte proliferation was evident in whole blood cultures both before and after the data had been normalized for the number of lymphocytes in culture indicating that the suppressive effect of the drug treatments on whole blood lymphocyte proliferation was not simply due to a reduced number of circulating lymphocytes. This is consistent with our previous findings with MDMA where lymphocyte proliferation was assessed using isolated peripheral blood mononuclear cells adjusted to a standard cell concentration in culture (Connor et al., 1998, 1999a,b). Thus the data generated in the present study indicate that the non-psychostimulant amphetamine derivative FEN, shares the ability of the psychostimulant methylenedioxy-amphetamine derivatives to suppress these indices of immune

function in the rat. Thus it appears that the ability of these serotonin releasing agents to alter immune functioning occurs independently of their ability to produce behavioural activation.

With respect to Th1 and Th2 type cytokine production, the effects of the three amphetamine derivatives were similar prior to adjusting for lymphocyte numbers in the whole blood cultures, in that both IFN-γ and IL-10 production were decreased by all three amphetamine derivatives, with MDA (54% and 74%) and FEN (62% and 79%), being more potent than MDMA (20% and 52%) in this respect. In contrast, IL-2 production was not significantly altered by any of the drug treatments despite the fact that there was a 32–49% decrease in lymphocyte numbers in culture following drug treatments. However, when the cytokine production data were normalized for the number of lymphocytes in culture to give an insight to the effects of drug treatments on cytokine production at a cellular level, the effects of the three amphetamine derivatives began to diverge. All three drugs suppressed IL-10 production with MDMA being less potent than MDA and FEN in this respect. With respect to IL-2, both of the methylenedioxy-substituted amphetamines (MDA and MDMA) increased Con A-stimulated production of this Th1 type cytokine, whereas FEN did not significantly alter IL-2 production. There was also a differential effect of the three drugs on IFN-γ production, in that FEN provoked a potent suppressive effect on IFN-γ production but neither treatment with MDMA or MDA significantly altered IFN-γ secretion. Thus FEN shared the ability of MDA and MDMA to suppress Th2 type cytokine production as evidenced by the potent suppression of Con A-stimulated IL-10 production provoked by these drugs. In fact FEN was the most potent of the three drugs in this respect.

However, the effects of FEN on Th1 type cytokine production were very different to that of MDA and MDMA. While MDMA and MDA increased the production of IL-2 and did not alter IFN-γ production, FEN provoked a potent suppression of IFN-γ secretion without altering IL-2 secretion. Thus whilst the methylendioxy-substituted amphetamines increase Th1 type cytokine secretion at a cellular level as indicated by increased IL-2 secretion, FEN suppressed Th1 type cytokine secretion at a cellular level as indicated by its ability to inhibit IFN-γ production. Therefore the effects of these drugs on Th1 type cytokine secretion is complex, in that individual Th1 type cytokines (IL-2 and IFN-γ) are differentially affected by the drugs. The underlying basis of the differential effects of these drugs on cytokine secretion is not known, but is unlikely to be due to their differential effects on glucocorticoid secretion as all three drugs potently activate the HPA axis and increase circulating corticosterone concentrations (McGarvey, 1995). It should not be ruled out that in addition to the effects of these drugs on hormonal system in vivo, that either the parent compounds or drug metabolites may well exert direct actions on lymphocytes and alter their ability to produce cytokines. In this regard it was previously reported that MDMA has effects on a number of immune parameters when incubated in vitro (House et al., 1995). For example, in vitro incubation of T-cells with MDMA altered the pattern of IL-2 secretion in that a low concentrations of MDMA (0.0001 μM) enhanced IL-2 production, whereas higher concentrations either did not alter (0.001–10 μM), or suppressed (100 μM) IL-2 production from T-cells (House et al., 1995). In the present study both MDMA and its metabolite MDA increased IL-2 production at a cellular level. However, as the plasma concentrations of these drugs were not measured it is difficult to make comparisons between our data and those data of House et al. (1995). In addition to these amphetamine-derivatives having a direct effects on lymphocytes, it is known that these drugs release serotonin from platelet stores in the periphery (Buczko et al., 1975; Rudnick and Wall, 1992), thus peripherally released serotonin may have a direct effect on lymphocyte function as previously reported (Kut et al., 1992). The relative potencies of these drugs to release peripheral serotonin is not known, but it is possible that a differential effect on peripheral serotonin release could account for the differential effects of these drugs on lymphocyte proliferative responses and cytokine production profiles. However further studies are needed in order to investigate these possibilities.

The effects of drug treatments on macrophage activity were examined by measuring LPS-induced secretion of the proinflammatory cytokines IL-1β and TNF-α from whole blood cultures. The data indicate that all three drugs inhibit LPS-induced

TNF-α secretion but fail to significantly alter IL-1β secretion. In fact if anything, all three drugs tended to increase IL-1β secretion from LPS-stimulated whole blood cultures. The effect of drug treatments on TNF-α secretion suggest that macrophage activity is impaired following treatment with all three amphetamine derivatives. In support of this hypothesis preliminary data from our laboratory using an in vivo LPS challenge model in rats indicate that both MDMA and FEN potently suppress the ability of rats to produce IL-1β, IL-6 and TNF-α following LPS administration, with the suppression of TNF-α being the most profound and longlasting effect (Connor et al., 1999b; Unpublished data). Therefore the in vivo LPS challenge approach may yield more meaningful results than observed in the present study using ex vivo stimulation of whole blood cultures with LPS to assess macrophage function.

In conclusion, the data generated in the present study demonstrate that for all the immune parameters examined MDMA and MDA had qualitatively similar effects. However, for the most part MDA provoked more profound changes than MDMA. Similar to MDA and MDMA, FEN reduced the number of circulating lymphocytes, provoked a suppression of Con A-stimulated lymphocyte proliferation and total IFN-γ and IL-10 production in whole blood cultures. Thus the non-psychostimulant amphetamine derivative FEN shares the ability of the psychostimulant methylenedioxy-substituted amphetamine derivatives to alter these indices of immune function in the rat. However, when Con A-stimulated cytokine production was normalised for the number of lymphocytes in culture in order to examine cytokine production at a cellular level, the effect of the amphetamine derivatives begins to diverge. FEN shares with MDMA and MDA the ability to suppress production of the Th2 type cytokine IL-10. However the effect of these drugs on Th1 type cytokine secretion was much more complex. While the methylendioxy-substituted amphetamines increases the secretion of the Th1 type cytokine IL-2 without altering the related Th1 type cytokine IFN-γ, FEN did not alter IL-2 secretion, but suppressed IFN-γ secretion. In addition to the effect of these drugs on T-cell responses, all three drugs inhibited LPS-induced TNF-α secretion from whole blood cultures suggesting that macrophage activity is impaired following treatment with all three amphetamine derivatives. In all, these data extend our previous findings concerning the effects of MDMA on the immune system (Connor et al., 1998, 1999a,b) and demonstrate that the related serotonin releasers MDA and FEN also provoke alterations in immunity in the rat. However as very little is known about the functional reserve of the immune system further studies examining susceptibility to bacterial/viral infections are required in order to determine whether the drug-induced immunological changes observed in the present study have a major impact on infectivity and disease progression.

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