The effects of fumonisins on sphinganine and sphingosine levels in hepatocyte cultures, experimental animals and humans

Liana van der Westhuizen

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Promotor:	Prof. P. D. van Helden
Project leader:	Dr. G. S. Shephard

DECLARATION

I, the undersigned, hereby declare that the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Signature:

Date:

Abstract

Fumonisin mycotoxins, of which fumonisin B_1 (FB_1) and B_2 (FB_2) are the major metabolites, are produced by *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon), a fungus that occurs worldwide on maize. Fumonisins cause various syndromes in different animal species and high levels of fumonisins in maize have been correlated with the high incidence areas of human oesophageal cancer. As fumonisins occur widely around the world in maize products intended for human consumption, they pose a potential hazard to human health. Direct measurement of FB_1 in plasma as a biomarker of fumonisin exposure is not feasible as it is eliminated rapidly from plasma, has a low bioavailability and lacks a major metabolite. The primary target of the fumonisins in animal cells is ceramide synthase, a key enzyme in the *de novo* sphingolipid biosynthetic pathway. The inhibition of ceramide synthase leads to an elevation of the sphingoid base, sphinganine (Sa), in cells, thus resulting in an increase in the Sa/So ratio. An elevation in the Sa/sphingosine (So) ratio after exposure to fumonisins has previously been observed in serum, urine and various tissues in various animal species. As the changes in the Sa/So ratio occur before other biochemical markers of cellular injury, it has been proposed as a possible biomarker for fumonisin exposure.

The structure-activity studies in primary rat hepatocyte cultures showed that at least one tricarballylic acid (TCA) group is required for maximal ceramide synthase inhibition, that the presence of a free amino-group is not a requisite for enzyme inhibition and that the inhibition in primary rat hepatocytes was irreversible.

Even though a single low- or high- FB_1- or FB_2-dose is rapidly eliminated from plasma in vervet monkeys, the resulting elevation of the plasma Sa/So ratio is observed for an extended period after dosing. The effect of FB_2, which is less polar than FB_1, may be more persistent in its *in vivo* effects due to longer sustained increases. Repeated low-FB_1-doses resulted in more severe and sustained disruption of sphingoid metabolism than a single FB_1 dose at either level. Only the single high-FB_1-dose had an observable effect on the urinary Sa/So ratios, causing a brief transient increase. This would suggest that FB_2 may be less nephrotoxic in monkeys than FB_1 and therefore it appears that in monkeys the plasma Sa/So ratio is more effective as a biomarker of fumonisin exposure than the urinary Sa/So ratio.

No significant differences were found in the mean plasma and urinary Sa/So ratios between males and females nor between the combined ratios in human volunteers in Transkei and KwaZulu-Natal, South Africa and Bomet district, Kenya. However, the upper levels of the ranges of the Sa/So ratios in volunteers in the Transkei, where significantly higher levels of fumonisins were found in the maize, were much higher than the upper ranges of the ratios in the other two areas. The analysis of the data from the human volunteers from these areas in Africa suggests that the Sa/So ratio may be only of limited value as a biomarker of fumonisin exposure.

Opsomming

Fumonisien mikotoksiene, waarvan fumonisien B_1 (FB_1) en B_2 (FB_2) die belangrikste metaboliete is, word deur *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon), 'n fungus wat wêreldwyd op mielies voorkom, geproduseer. Fumonisiene veroorsaak verskeie sindrome in verskillende diere en hoë vlakke van fumonisiene op mielies is gekorreleer met hoë voorkoms van slukdermkanker in dieselfde areas. Siende dat fumonisiene so wyd verspreid voorkom op produkte wat bestem is vir menslike gebruik, is dit 'n potensiële gesondheidsrisiko. Direkte bepaling van FB_1 in plasma as 'n biomerker van fumonisien blootstelling is nie haalbaar nie, omdat dit vinnig afgebreek word in plasma en dit 'n lae biobeskikbaarheid het en dit het nie 'n hoof metaboliet nie. Die primêre teiken van fumonisiene in dierselle is seramied sintese, 'n sleutel ensiem in die *de novo* sfingolipied biosintese weg. Die inhibisie van seramied sintese lei tot 'n verhoging in die sfingoid basis, sfinganien (Sa), in selle wat veroorsaak dat die Sa/sfingosien (So) verhouding toeneem. 'n Verhoging in die Sa/So verhouding na blootstelling aan fumonisiene is reeds in serum, uriene en verkeie weefsels in verskillende diere waargeneem. Omdat die veranderinge in die Sa/So verhouding geskied voor die verandering in ander biochemiese merkers van selbeskadiging, is dit voorgestel as 'n moontlike biomerker van fumonisien blootstelling.

Struktuur studies in primêre rot hepatosiet kulture het gewys dat minstens een trikarboksielsuur groep noodsaaklik is vir maksimale seramied sintese inhibisie, dat die teenwoordigheid van 'n vry amino-groep nie noodsaaklik is nie en dat die ensiem inhibisie onomkeerbaar is.in primêre rot hepatosiet kulture.

Alhoewel 'n enkele lae- of hoë-FB_1- of FB_2-dosis vinnig in plasma van blou ape geëlimineer. word, word die effek van die verhoging van die Sa/So verhouding in plasma vir 'n geruime tyd na dosering bespeur. Die effek van FB_2, wat minder polêr is as FB_1, is dalk van langer duur in die *in vivo* effekte as gevolg van die langer volgehoue verhoging. Herhaalde lae-FB_1-dosisse veroorsaak erger and langer volgehoue versteurings in sfingoid metabolisme as 'n enkele FB_1 dosis. Slegs die enkele hoë- FB_1-dosis het 'n merkbare effek van verbygaande aard in uriene getoon. Dit blyk dus dat FB_2 minder nefroties in ape is as FB_1 en dat minstens in ape die plasma Sa/So verhouding meer effektief is as 'n biomerker van fumonisien blootstelling as die urien Sa/So verhouding.

Geen betekinsvolle verskille is gevind tussen die gemiddelde plasma en urine Sa/So verhoudings van mans en vrouens of tussen die gekombineerde verhoudings van die vrywilligers van Transkei en KwaZulu-Natal, Suid-Afrika en Bomet, Kenya nie. Die boonste vlak van die reeks van die Sa/So verhouding in die vrywilligers is betekenisvol hoër in Transkei waar hoë fumonisien vlakke in die mielies gevind is as in die ander twee areas. Uit die analise van die data van hierdie areas van Afrika blyk dit dat die Sa/So verhouding slegs van beperkte belang mag wees as 'n biomerker van fumonisien blootstelling.

Opgedra aan my ouers

Albie en Ella van der Westhuizen

CONTENTS

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General introduction

Abbreviations: AAL = *Alternaria* toxins; AP_1 = aminopentol; FA_1 = Fumonisin A_1; FB_1 = Fumonisin B_1; FB_2 = Fumonisin B_2; Sa = Sphinganine; So = Sphingosine; TA and TB = AAL toxin analogues.

Fumonisin B_1 (FB_1) and B_2 (FB_2) are the major mycotoxins produced by *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon) as secondary metabolites. *F. verticillioides* is a seed-borne fungus which is widely associated with maize intended for human and animal consumption (Shephard *et al.*, 1996a). Fumonisins cause various syndromes in different animal species such as leukoencephalomalacia in horses (Marasas *et al.,* 1988; Kellerman *et al.*, 1990), pulmonary oedema in pigs (Harrison *et al.,* 1990) and hepatocarcinoma in rats (Gelderblom *et al.,* 1991). High levels of fumonisins have been found in maize from the high incidence areas of oesophageal cancer in Transkei, South Africa (Rheeder *et al.*, 1992) as well as in the Cixian and Linxian counties of China (Chu and Li, 1994). An association between consumption of maize highly contaminated with fumonisin and liver cancer in humans in China has also been suggested (Ueno *et al.,* 1997). Based on existing data, the International Agency for Research on Cancer (IARC) has declared "toxins derived from *F. moniliforme*" to be possibly carcinogenic to humans (class 2B carcinogens) (IARC, 1993). As fumonisins occur widely around the world in maize products intended for human consumption, it raised concern over the potential hazard to human health posed by these compounds (Shephard *et al.*, 1996a; Thiel *et al.*, 1992), especially in the developing world where

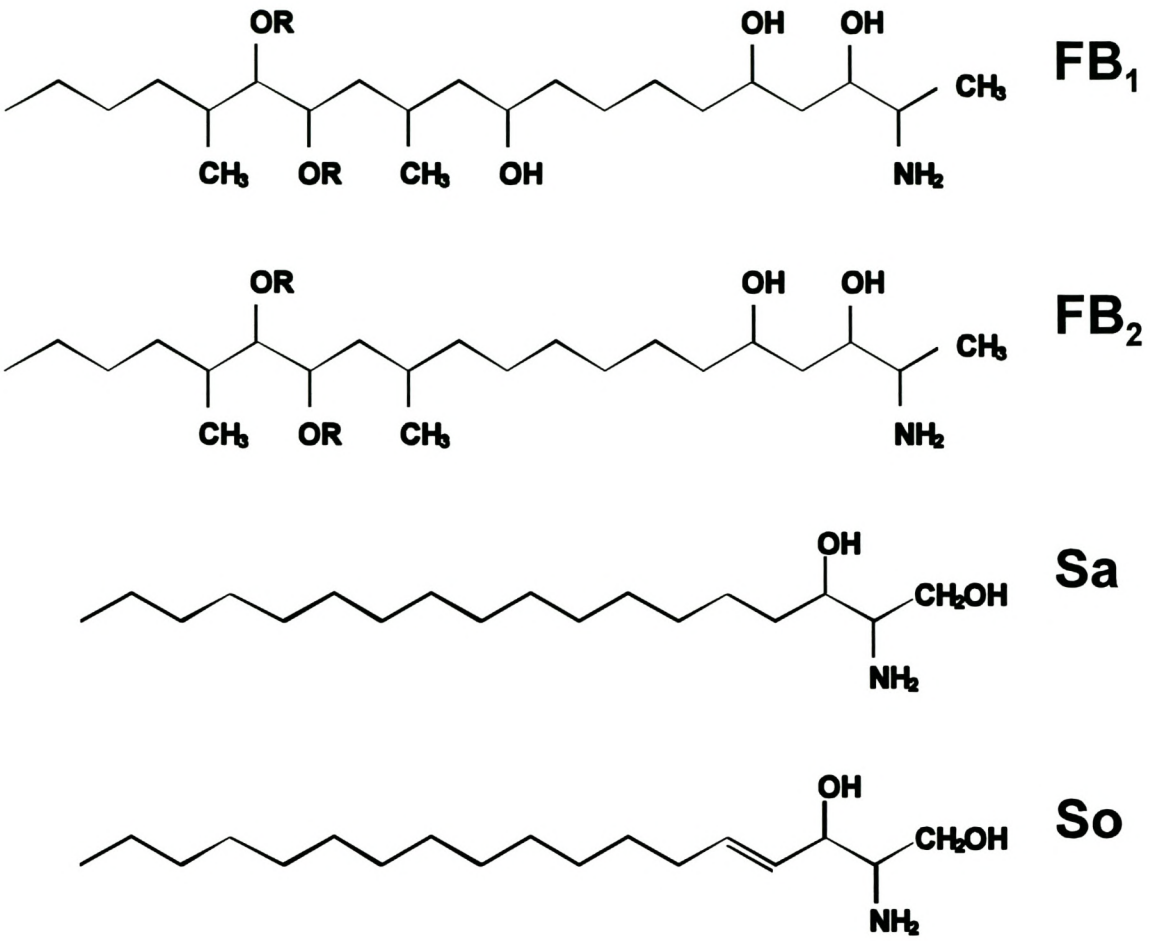

Fig. 1. *Chemical structures of fumonisin B_1, fumonisin B_2, sphinganine and sphingosine.*

populations rely on maize base foods as their staple diet and these populations can be chronically exposed to highly contaminated food.

Sphingolipids are structurally composed of a long-chain sphingoid base, an amide-linked fatty acid and a polar head group at the 1-position and consist of a large group of chemical structures that include the free long-chain bases, sphinganine (Sa) and sphingosine (So), ceramides, glycosphingolipids, sphingomyelin, phosphosphingolipids and other compounds including membrane anchors for proteins (Merrill, 1991). Sphingolipids constitute a class of membrane lipids that play an important role in cell regulation defining the structural properties of membranes and lipoproteins, have important roles in cell-cell and cell-substratum interaction and in other forms of cell recognition and help regulate cell growth and differentiation (Merrill *et al.* 1997).

Direct measurement of fumonisins in plasma is not feasible as it is eliminated rapidly, has a low bioavailability and lacks a major metabolite in experimental animals (Shephard *et al.*, 1998). The primary target of the fumonisins in animal cells is So (Sa) *N*-acyltransferase (ceramide synthase) (Wang *et al.,* 1991), a key enzyme in the *de novo* sphingolipid biosynthetic pathway (see Fig.1). Ceramide synthase catalyses the conversion of Sa to dihydroceramide, which is then converted to ceramide via addition of the 4,5-trans double bond. Ceramide undergoes further metabolism to produce complex sphingolipids, such as glycosphingolipids and sphingomyelin (Merrill, 1991), while So is produced as the turnover product of ceramide and other complex sphingolipids. So can be re-acylated by ceramide synthase back to ceramide or

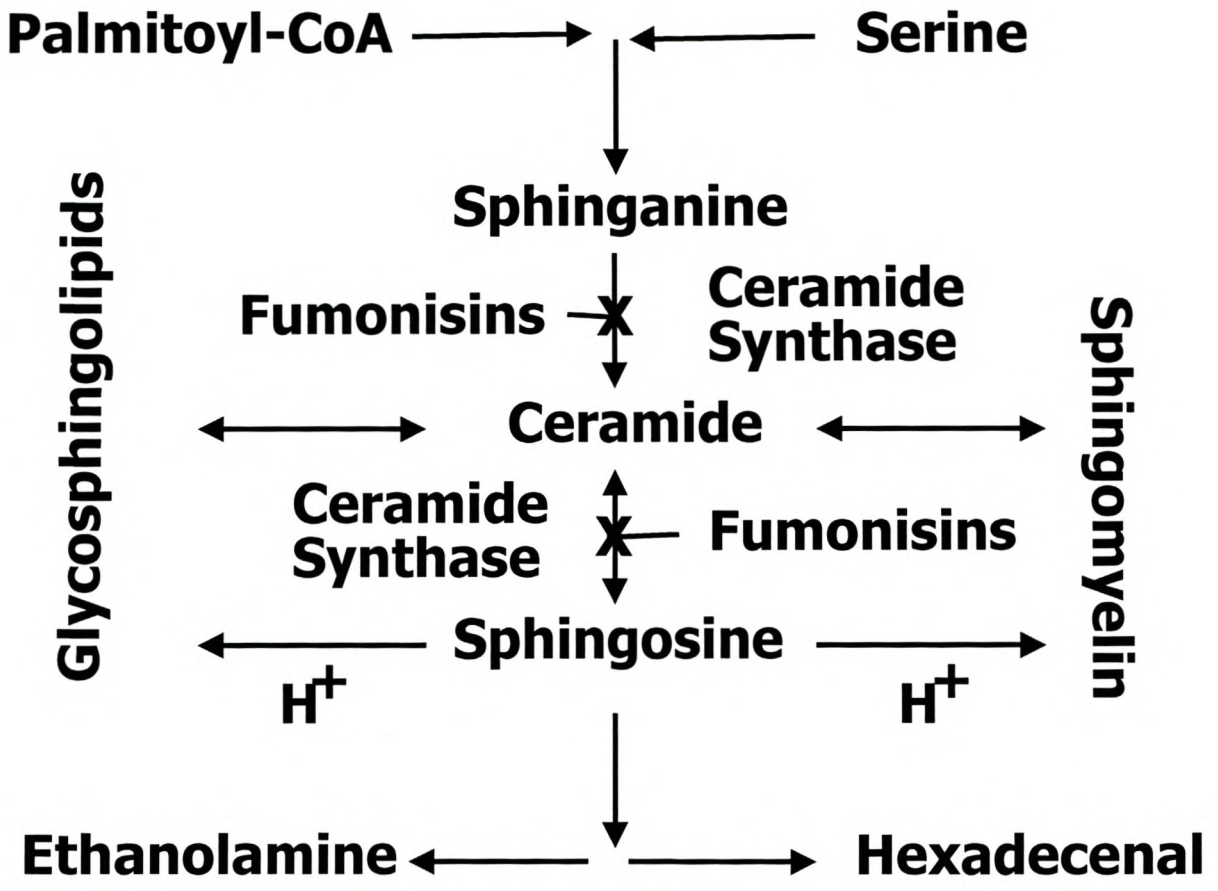

Fig. 2. *A schematic presentation of the de novo sphingolipid biosynthetic and the sphingolipid turnover pathway showing the inhibition of fumonisins on the ceramide synthase enzyme, blocking the acylation of sphinganine and thus preventing the formation of ceramide and also preventing the reconversion of sphingosine to ceramide.*

undergoes further catabolism (Rother *et al.*, 1992). Fumonisins inhibit ceramide synthase and cause the accumulation of Sa, and So to a lesser extent, in cells (Wang *et al.*, 1991) and decreased complex sphingolipid levels (Wang *et al.*, 1992, Riley *et al.*, 1997). The inhibition of ceramide synthase and the resultant increase in Sa/So ratio is specific to fumonisins and fumonisin-like toxins (Norred *et al.*, 1997). As sphingoid bases and ceramide play important roles in cell cycle progression and apoptosis (Merrill *et al.*, 1997), the disruption of the sphingolipid pathway, caused by fumonisin inhibition, can affect carcinogenesis (Ciacci-Zanella *et al.*, 1998).

The effect of fumonisins on the Sa/So ratios in serum and/or urine and/or various tissues (liver, kidney, lung, brain, muscle) has been investigated in various animal species: rats, mice, pigs, horses, rabbits, vervet monkeys, broiler chicks, turkey poults, mink, catfish and rainbow trout (Castegnaro *et al.*, 1996; 1998; Gelderblom *et al.*, 1996a; 1996b 1996c; 1997; Goel *et al.*, 1994; 1996; Gumprecht *et al.*, 1995; Gurung *et al.*, 1998; Haschek *et al.*, 1992; Laborde *et al.*, 1997; Ledoux *et al.*, 1996; Martinova and Merrill, 1995; Meredith *et al.*, 1998; Morgan *et al.*, 1997; Reddy *et al.*, 1995; 1996; Restum *et al.*, 1995; Riley *et al.*, 1993; 1994a; 1997; Rotter *et al.*, 1996; Shephard *et al.*, 1996b; Tsunoda *et al.*, 1998; Van der Westhuizen et al., 1999; Voss *et al.*, 1995; 1996; 1998; Wang *et al.*, 1992; 1999; Weibking *et al.,* 1994). The free sphingoid bases in urine arise from the cells that accumulate in urine (Riley *et al.*, 1994a), whereas it is uncertain from which particular tissues those in serum arise as most, if not all, tissues appear to have the capacity for sphingoid base biosynthesis (Merrill *et al.*, 1993). As these changes occur before other biochemical markers of cellular injury, it has been

proposed that the Sa/So ratio could be a possible biomarker for fumonisin exposure (Wang *et al.*, 1992; Riley *et al.*, 1993; 1994b).

Structural differences within the fumonisin group of mycotoxins have been utilised to study structure-activity relationships with respect to specific biological effects (Abbas *et al.*, 1993; 1994; Gelderblom *et al.*, 1993; Shier *et al.*, 1991). The AAL toxins, of which TA and TB are the main forms, are structurally related phytotoxins produced by *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici*, a fungus that causes stem canker disease in certain susceptible tomato cultivars (Bottini *et al.*, 1981; Gilchrist and Grogan, 1976; Shephard *et al.*, 1993). AAL toxins (Merrill *et al.*, 1993), as in the case of fumonisins (Wang *et al.*, 1991), inhibit ceramide synthase. AAL toxin and FB_1 also disrupt sphingolipid biosynthesis in plants (Abbas *et al.*, 1995). FB_1, as well as TA and TB, have been shown to be cytotoxic to certain mammalian cell lines. There were variations in sensitivities to fumonisins and AAL toxins among cell lines tested, depending on the tissue of origin and possibly the degree of differentiation (Shier *et al.*, 1991). When monitoring the inhibitory effect on cell proliferation in Madin-Darby canine kidney (MDCK) cells and a rat hepatoma cell line (H4TG), FA_1 (*N*-acetyl derivative of FB_1) exhibited little or no activity, while AP_1 (a hydrolysis product of FB_1) showed similar or greater effects than FB_1, FB_2 and fumonisin B_3 (FB_3) (Abbas *et al.*, 1993). In primary hepatocytes, FA_1 exhibited a lower and AP_1 a higher cytotoxicity, than FB_1 and FB_2 (Gelderblom *et al.*, 1993). In plants FB_1 and TA caused higher leaf necrotizing activity on detached tomato leaves than FA_1 and AP_1 (Lamprecht *et al.*, 1994). FB_1, FB_2 and FB_3 exhibited cancer-initiating activity in an *in vivo* cancer initiating/promoting model in

rat liver, while AP_1 and FA_1 lacked activity (Gelderblom *et al.*, 1993). TA is cytotoxic to certain rat and dog tissue culture cells, whereas its *N*-acetylated analogue is not (Mirocha *et al.*, 1992). At present it is not known whether biological effects similar to those occurring with the fumonisins could be induced *in vivo* by the AAL toxins in the different animal species (Abbas *et al.*, 1994). Certain *in vitro* biological effects such as cytotoxicity and phytotoxicity are known to be similar.

In chapter 1 the structural requirements for ceramide synthase inhibition were investigated by comparing the effect of structurally related compounds (FB_1, FA_1, AP_1, TA and TB) on the Sa and So concentrations and hence the Sa/So ratio in rat primary hepatocyte cultures. The extent to which sphingolipid biosynthesis was affected was correlated with the respective cytotoxicities of the structural analogues *in vitro*. The reversibility of the inhibitory effect was investigated to obtain more information about the biological significance of sphingolipid inhibition in primary hepatocytes.

In chapter 2 the effect of a single gavage dose of either 1 or 10 mg FB_1 /kg body weight was monitored in vervet monkeys (*Cercopithecus aethiops*) for seven days, where after the primates were sacrificed. The Sa and So levels were determined daily in serum and urine of the monkeys and, on termination of the experiment, also in liver and kidney tissues. Serum cholesterol and serum levels of certain liver function enzymes [alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transferase (GGT) and lactate dehydrogenase (LDH)] were monitored. Renal function was monitored by measuring urea and creatinine levels in the serum. As the parameters monitored in the

initial study had not returned to baseline levels within seven days, a further similar study was undertaken in which monkeys were given identical doses of FB_1 and monitored until all parameters returned to normal (36 - 50 days). These monkeys were not terminated at the end of the study.

In chapter 3 the effects on the levels of Sa and So of repeated doses (three times /week) at 1 mg FB_1 /kg body weight or single gavage doses of either 1 or 10 mg FB_2 /kg body weight in vervet monkeys were monitored over a 51 day period. In addition plasma cholesterol and plasma levels ALT, AST, GGT, LDH, urea and creatinine were monitored.

In chapter 4 the aim was to determine the Sa/So ratio in plasma and urine of maize-consuming rural populations and was undertaken in the Centane (Kentani) district, Transkei region of the Eastern Cape province and Madadeni district, northern KwaZulu-Natal province of South Africa, as well as in the Bomet district, western Kenya. Sa and So levels were determined in the plasma and urine of male and female volunteers, while the levels of fumonisins were determined in maize samples collected contemporaneously from these regions.

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Chapter 1

The inhibition of sphingolipid biosynthesis in rat primary hepatocyte cultures by fumonisin B_1 and other structurally related compounds

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ABSTRACT

The fumonisins and toxins produced by *Alternaria alternata* f. sp. *lycopersici* (AAL toxins) are structurally related mycotoxins that disrupt sphingolipid biosynthesis by inhibiting the rate limiting enzyme, ceramide synthase. Rat primary hepatocytes were exposed to fumonisin B_1 (FB_1), its N-acetyl analogue, FA_1, its fully hydrolysed analogue, AP_1 and the AAL toxins (TA and TB) at concentrations of 1 µM for 40 hr in culture. The extent to which these compounds disrupt sphingolipid biosynthesis in hepatocytes *in vitro* was investigated by analysing the sphingosine (So) and sphinganine (Sa) levels by HPLC. The inhibition of ceramide synthase was irreversible as the Sa/So ratio was maximally increased by FB_1 after 24 hr of exposure and the subsequent removal of FB_1 had no effect on the ratio as compared to the 40 hr incubation period in the presence of FB_1. The Sa concentration was significantly ($p<0.01$) increased in all the cultures treated with the different structurally related compounds, while only AP_1 increased the So concentration significantly ($p<0.05$) above the control. As AP_1 was found to be less effective in disrupting sphingolipid biosynthesis it would appear that the tricarballylic (TCA) moiety is required for maximal inhibition of ceramide synthase. The presence of an amino-group appears not to be a requisite for activity, as FA_1 increased the Sa/So ratio to the same extent as FB_1. The AAL toxins, TA and TB increased the Sa concentration significantly ($p<0.01$) above that of FB_1 and FA_1, while the Sa/So ratios were altered to the same extent. The structural requirements for the induction of cytotoxicity differ from those required for ceramide synthase inhibition as TA and TB were significantly ($p<0.05$ to $p<0.01$) less toxic to primary hepatocytes than FB_1 at all

the concentrations tested.

Abbreviations: AP_1 = aminopentol; DMSO = Dimethyl sulphoxide; LDH = lactate dehydrogenase; FA_1 = Fumonisin A_1; FB_1 = Fumonisin B_1; FB_2 = Fumonisin B_2; OPA = *o*-phthaldialdehyde; So = Sphingosine; Sa = Sphinganine; TCA = Tricarballylic acid.

INTRODUCTION

Fumonisins are mycotoxins produced by *Fusarium moniliforme* Sheldon, a fungus that occurs worldwide on maize (Shephard *et al.*, 1996). Fumonisin B_1 (FB_1) (fig. 1) is the most abundant of the various analogues that have been isolated (Shephard *et al.*, 1996). FB_1 causes various syndromes in different animal species: leukoencephalomalacia in horses (Marasas *et al.*, 1988, Kellerman *et al.*, 1990), pulmonary oedema in pigs (Harrison *et al.*, 1990) and hepatocarcinoma in rats (Gelderblom *et al.*, 1991). Although the occurrence of the fumonisins has been statistically associated with a high incidence of human oesophageal cancer in southern Africa (Rheeder *et al.*, 1992), evidence for a contributing role in the development of this disease in experimental animals is lacking. The AAL toxins, of which TA and TB are the main forms (fig. 1), are structurally related phytotoxins produced by *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici*, a fungus that causes stem canker disease in certain susceptible tomato cultivars (Bottini *et al.*, 1981, Gilchrist and Grogan, 1976, Shephard *et al.*, 1993). AAL toxin (TA) is cytotoxic to certain rat and dog tissue culture cells, whereas its

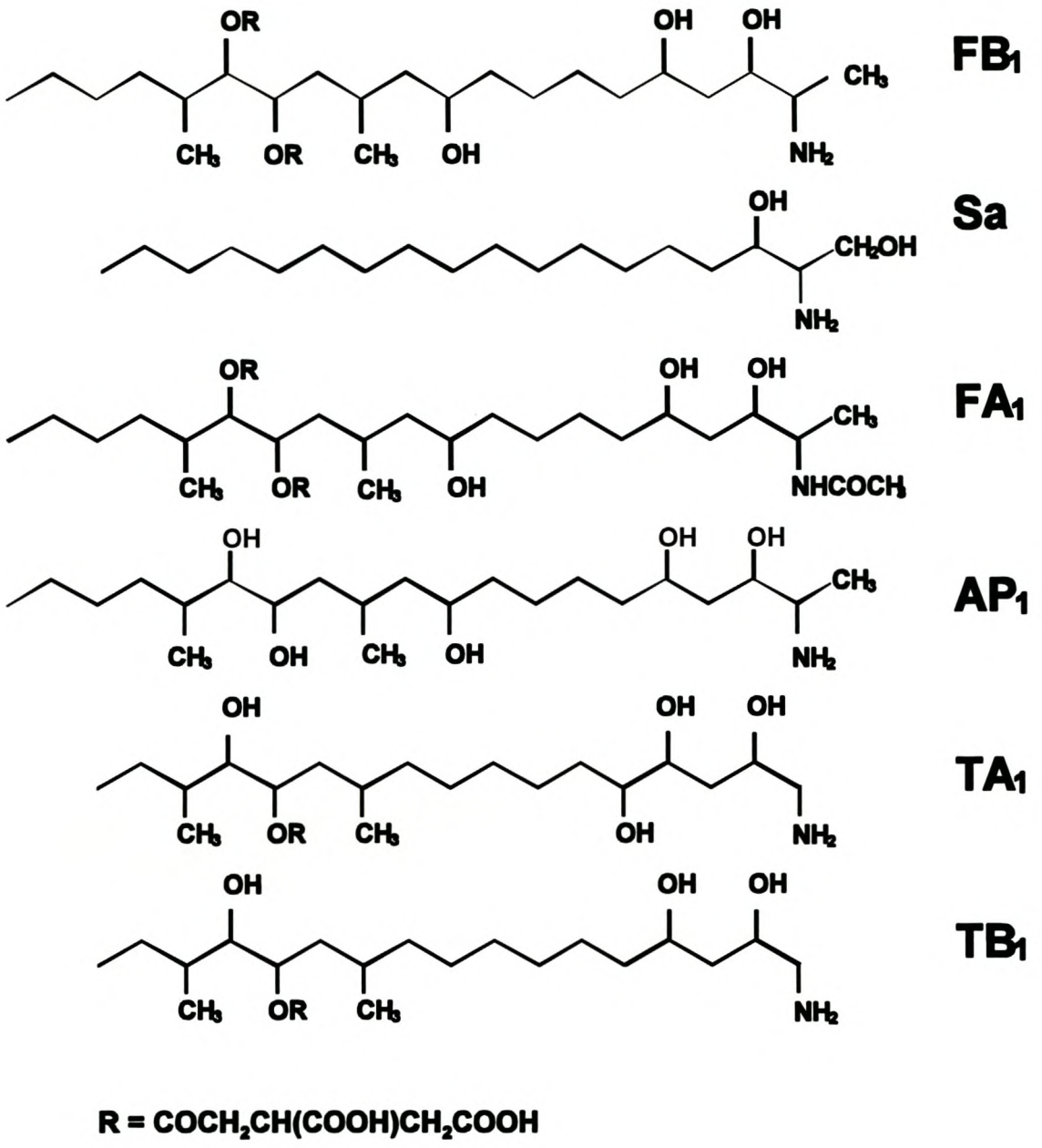

Fig. 1. *Chemical structures of fumonisin B_1, sphinganine, fumonisin A_1, AP_1, the hydrolysis product of FB_1, and the individual isomers of the AAL toxins, designated TA_1 and TB_1. The isomers esterified at C_{14} are called TA_2 and TB_2 (see Materials and Methods).*

N-acetylated analogue is not (Mirocha *et al.*, 1992). At present it is not known whether biological effects similar to those occurring with the fumonisins could be induced *in vivo* by the AAL toxins in the different animal species (Abbas *et al.*, 1994). Certain *in vitro* biological effects such as cytotoxicity and phytotoxicity are known to be similar.

Structural differences within the fumonisin group of mycotoxins have been utilised to study structure-activity relationships with respect to specific biological effects (Abbas *et al.*, 1993, 1994, Gelderblom *et al.*, 1993, Shier *et al.*, 1991). FB_1, as well as TA and TB, have been shown to be cytotoxic to certain mammalian cell lines. There were variations in sensitivities to fumonisins and AAL toxins among cell lines tested, depending on the tissue of origin and possibly the degree of differentiation (Shier *et al.*, 1991). When monitoring the inhibitory effect on cell proliferation in Madin-Darby canine kidney (MDCK) cells and a rat hepatoma cell line (H4TG), FA_1 (*N*-acetyl derivative of FB_1) exhibited little or no activity while AP_1 (a hydrolysis product of FB_1) showed similar or greater effects than FB_1, fumonisin B_2 (FB_2) and fumonisin B_3 (FB_3) (Abbas *et al.*, 1993). In primary hepatocytes FA_1 exhibited a lower and AP_1 a higher cytotoxicity than FB_1 and FB_2 (Gelderblom *et al.*, 1993). In plants FB_1 and TA caused higher leaf necrotizing activity on detached tomato leaves than FA_1 and AP_1 (Lamprecht *et al.*, 1994). FB_1, FB_2 and FB_3 exhibited cancer initiating activity in an *in vivo* cancer initiating/promoting model in rat liver, while AP_1 and FA_1 lack activity (Gelderblom *et al.*, 1993).

Fumonisins (Wang *et al.*, 1991) and AAL toxins (Merrill *et al.*, 1993b) inhibit sphingosine

(So) [sphinganine (Sa)] *N*-acyltransferase (ceramide synthase), a key enzyme in the sphingolipid biosynthetic pathway in animal cells. The inhibition of this enzyme leads to an elevation of Sa and So levels in cells, although Sa levels rise to a much greater extent than the So levels, thus resulting in an increase in the Sa/So ratio (Riley *et al.*, 1994). FB_1 disrupted the sphingolipid profiles in the following cell culture systems: rat primary hepatocytes (Wang *et al.*, 1991, Gelderblom *et al.*, 1995), a renal epithelial cell line, LLC-PK_1 (Yoo *et al.*, 1992), Swiss 3T3 fibroblasts (Schroeder *et al.*, 1994) and mouse cerebellar neurons (Merrill *et al.*, 1993a). AAL toxin and FB_1 also disrupt sphingolipid biosynthesis in plants (Abbas *et al.*, 1995).

In this study the structural requirements for ceramide synthase inhibition was investigated by comparing the effect of structurally related compounds (FB_1, FA_1, AP_1, TA and TB) on the Sa/So ratio as well as the Sa and So concentrations in rat primary hepatocyte cultures. The extent to which sphingolipid biosynthesis was affected was correlated with the respective cytotoxicities of the structural analogues *in vitro*. The reversibility of the inhibitory effect was investigated to obtain more information about the biological significance of sphingolipid inhibition in primary hepatocytes.

MATERIALS AND METHODS

Mycotoxin standards and chemicals

FB_1, FA_1 and AP_1 were purified as described previously by Cawood *et al.* (1991) and

Gelderblom *et al.* (1993). The chemical purity of the structural analogues were determined by ^{13}C NMR, HPLC and TLC as described by Cawood *et al.* (1991). TA and TB were purified as described by Shephard *et al.* (1993) and, in solution, consisted of a natural equilibrium of 2 isomers where either the C-13 (TA_1 and TB_1) or the C-14 (TA_2 and TB_2) hydroxyl group is esterified. Solutions of the individual toxins were prepared either in saline for FB_1, TA and TB or in dimethyl sulphoxide (DMSO) /saline (1:1) for AP_1 and FA_1. Sa and So were obtained from Sigma Chemical Company (St. Louis, MO, USA). C_{20}-Sa was a generous gift from Prof. A. H. Merrill Jr, Department of Biochemistry, Emory University School of Medicine, Atlanta, GA, USA.

Preparation of hepatocyte cultures

Primary hepatocytes were prepared from male Fischer 344 rats, weighing 150-200 g, by a collagenase perfusion technique (Hayes *et al.*, 1984). The viability of the hepatocyte preparations varied between 90 to 95 % when using trypan blue exclusion. The cells were seeded (6 x 10^5 cells per plate; 60 mm) in collagen-coated dishes in triplicate for 3 hr in modified Williams' E medium containing fetal bovine serum (10%), insulin (20 U/l), L-glutamine (2 mM), HEPES [N-(2-hydroxyethyl) piperazine-N'-(2-ethane sulphonic acid)] (10 mM), penicillin (100 U/ml) and streptomycin (100 µg/ml). The cells were washed with Hank's buffer solution and supplemented with serum-free, modified Williams' E medium containing L-proline (2 mM) and sodium pyruvate (10 mM) and incubated at 37°C. The treated plates and the controls (except the 0 hr control) were incubated for 40 hr.

Sa/So ratios in rat primary hepatocyte cultures

The plated cells were washed with ice-cold saline (2 ml; 3 times) prior to being harvested (0.5 ml saline; 3 times) by scraping with a rubber policeman. An aliquot (0.1 ml) was removed for protein determination (Kaushal and Barnes, 1986). The Sa and So concentrations were determined by HPLC with C_{20}- Sa as an internal standard, according to the method of Riley *et al.* (1994) with minor modifications. The lipids were extracted from the remainder of the hepatocyte cell suspension by incubation with methanol : chloroform (2:1) (containing 0.01% butylated hydroxytoluene as an antioxidant) under nitrogen at 37°C for 1 hr. Thereafter the mixture was washed twice with alkaline water, the phases separated by centrifugation and the chloroform fraction dried under nitrogen gas below 40°C. The residue was hydrolysed to release the free So by redissolving it in 0.1 M methanolic potassium hydroxide : chloroform (4:1), and incubated at 37°C for 1 hr. After washing with alkaline water, the chloroform phase was dried under nitrogen below 40°C.

HPLC quantification

The dried residues were stored at - 20°C overnight. Prior to analysis, the residues were re-dissolved in 250 µl methanol, sonicated and derivatised with 50 µl *o*-phthaldialdehyde (OPA) reagent as previously described (Riley et al. 1994). A 25-75 µl aliquot was injected into the HPLC which consisted of a Waters (Milford, MA, USA) Model 510 solvent delivery system, Waters U6K injector, Waters Radial-Pak™ cartridge packed with Nova-Pak C_{18} (4 µm, 100 x 8 mm), Autochrõm APEX Integration Chromatography Workstation and Perkin-Elmer (Norwalk, CT, USA) 650 S fluorescence

detector (excitation 335 nm and emission 440 nm). The isocratic mobile phase of methanol:0.005 M potassium phosphate buffer, pH 7.0 (91:9) was pumped at a flow rate of 2 ml/min.

Treatment of hepatocyte cultures

(1) FB_1

The hepatocyte control and treated cultures were incubated in triplicate. The control cultures, 0 hr (harvested before incubation) and 40 hr, were incubated with media containing saline without FB_1. The treatment groups were incubated as described above with media containing 1 µM FB_1 for 12 hr, 24 hr and 40 hr, respectively. After the initial12 hr and 24 hr period, respectively, the media of the corresponding culture groups containing the FB_1, were replaced with media without FB_1. All the treatment groups were incubated for 40 hr in total.

(2) Structurally related compounds

The hepatocyte control and treated cultures were incubated in triplicate. The hepatocyte control cultures were incubated in media with either saline or DMSO/saline (1:1). The primary hepatocytes were incubated in media containing 1 µM each of the mycotoxins FB_1, FA_1, AP_1, TA and TB for 40 hr.

Cytotoxicity determination

Detailed studies on the comparative cytotoxicity of AP_1, FA_1, FB_1, FB_2 and FB_3 have been published elsewhere (Gelderblom *et al.*, 1993). In the present study the

cytotoxicity of different concentrations (75, 250 and 500 μM) of FB_1, TA and TB were compared over a 40 h incubation period. The release of lactate dehydrogenase (LDH) in the culture medium was monitored by the method of Hayes *et al.* (1984). Cytotoxicities of the compounds were expressed as LDH release in the medium calculated as a percentage of the total LDH release in the control cells after treatment with Triton X100.

Statistical analysis

All the data were subjected to analysis of variance (ANOVA; 1-way), while the Tukey test was used to determine the statistical differences between means of the different treatment groups.

RESULTS

The effect of FB_1 on sphingolipid biosynthesis (Table 1)

Relatively small, but statistically significant, increases in the Sa concentration ($p<0.01$), So concentration ($p<0.05$) and Sa/So ratio ($p<0.05$) were observed between the 0 hr and 40 hr control hepatocyte cultures. In the hepatocytes incubated with FB_1 for 12, 24 and 40 hr the Sa level increased significantly ($p<0.01$) over the controls with a maximum accumulated at 24 hr. The corresponding So levels were significantly decreased ($p<0.01$ at 12 and 24 hr; $p<0.05$ at 40 hr) and therefore the Sa/So ratios were significantly ($p<0.01$) increased over the control after 40 hr of incubation. Within

the treated groups the maximal change in the Sa/So ratio ($p<0.05$, compared to 12 and 40 hr period) was obtained at 24 hr, while there was no significant difference in the ratios between the 12 and 40 hr exposure treatments.

Table 1. *The effect of FB_1 exposure, for various time intervals, on Sa and So concentrations in rat primary hepatocyte cultures after a 40 hr incubation period.*

Treatment	Sphinganine (Sa) (pmol/mg protein)	Sphingosine (So) (pmol/mg protein)	Ratio (Sa/So)
Control (40 hr)	1.73 ± 0.27 a	16.5 ± 1.8 a	0.11 ± 0.02 a
Control (0 hr)	0.56 ± 0.08 B	12.7 ± 0.4 b	0.04 ± 0.01 b
FB_1 (12 hr)	199.3 ± 9.9 C	11.7 ± 0.8 C	17.1 ± 0.7 C
FB_1 (24 hr)	249.7±14.2 D	11.6 ± 1.0 C	21.8 ± 3.0 D
FB_1 (40 hr)	224.7± 3.1 C	13.1 ± 1.2 c	17.4 ± 1.5 C

Values represent means ± SD of triplicate determinations. Control (40 hr) was separately compared with control (0 hr) and with the individual FB_1 treatment groups (12 hr, 24 hr and 40 hr). Values in a column followed by different letters (small and capital cases) differ significantly ($p<0.05$) from the control (40 hr); if both the letters and the cases differ, then $p<0.01$. Values followed by the same letter (small or capital cases) do not differ significantly ($P>0.05$).

The effect of structurally related compounds (Table 2)

There were no marked differences in the So concentrations and Sa/So ratio profiles of the control hepatocyte cultures incubated for 40 hr with the carrier solvents, saline or DMSO/saline (1:1), while the Sa levels decreased significantly ($p<0.01$) with the DMSO/saline (1:1) as the carrier solvent. In comparing the different compounds where saline was the solvent, the Sa levels increased significantly ($p<0.01$) over the control

value in the order TB>TA>FB_1 ($p<0.01$, between the individual treatment groups). There were no significant differences ($p>0.05$) in the So level in the saline group. The resulting Sa/So ratio increased significantly ($p<0.01$) over the control with no significant difference between the ratios for FB_1, TA and TB, due to the increase in the corresponding So concentrations. Within the group of analogues (AP_1 and FA_1) with DMSO/saline (1:1) as solvent, a significant ($p<0.01$) increase in the concentration of So was obtained with AP_1, and the Sa concentration and Sa/So ratio of AP_1 and FA_1 increased significantly ($p<0.01$) over the control.

Table 2. *The effect of structurally related compounds on sphingolipid profiles.*

Treatment	Sphinganine (Sa) (pmol/mg protein)	Sphingosine (So) (pmol/mg protein)	Ratio (Sa/So)
Hepatocytes incubated with saline			
Control (saline)	1.73 ± 0.27 a	16.5 ± 1.8 a	0.11 ± 0.02 a
FB_1	224.7 ± 3.1 b	13.1 ± 1.2 a	17.4 ± 1.5 b
TA	312.4 ± 9.7 c	19.0 ± 0.8 a	16.6 ± 0.2 b
TB	357.9 ± 10.3 d	25.2 ± 11.2 a	16.0 ± 5.7 b
Hepatocytes incubated with DMSO/saline (1:1)			
Control (DMSO/saline)	0.96 ± 0.03 c	13.6 ± 1.2 b	0.07 ± 0.01 c
FA_1	305.1 ± 10.2 dA	23.1 ± 0.1 b	13.3 ± 0.5 d
AP_1	153.5 ± 18.8 eB	37.8 ± 10.5 c	4.2 ± 0.6 e

Values represent means ± SD of triplicate determinations. Statistical comparisons between control and individual toxins were made within the different groups using saline and DMSO/saline as the control solvents. Values in a column followed by the same letter are not significantly different from the control ($p>0.05$), if the letter differs (capital cases) then $p<0.05$, if the letters (small cases) differ then $p<0.01$.

In comparing the AP_1 and FA_1 data with the results of the other analogues, FA_1 exhibits a similar effect on the Sa levels as compared to FB_1, TA and TB, although it was significantly higher than FB_1 ($p<0.01$) and lower ($p<0.01$) than TB. The Sa level of AP_1 was significantly lower ($p<0.01$) as compared to the other compounds, while the So level of AP_1 increased significantly ($p<0.05$) above those of the other treatments. Hence, the mean Sa/So ratio increased from the average baseline value of 0.1 to 4.2 after exposure to AP_1, which was significantly ($p<0.01$) lower as compared to the mean value of 15.8 obtained after exposure to FB_1, FA_1, TA and TB.

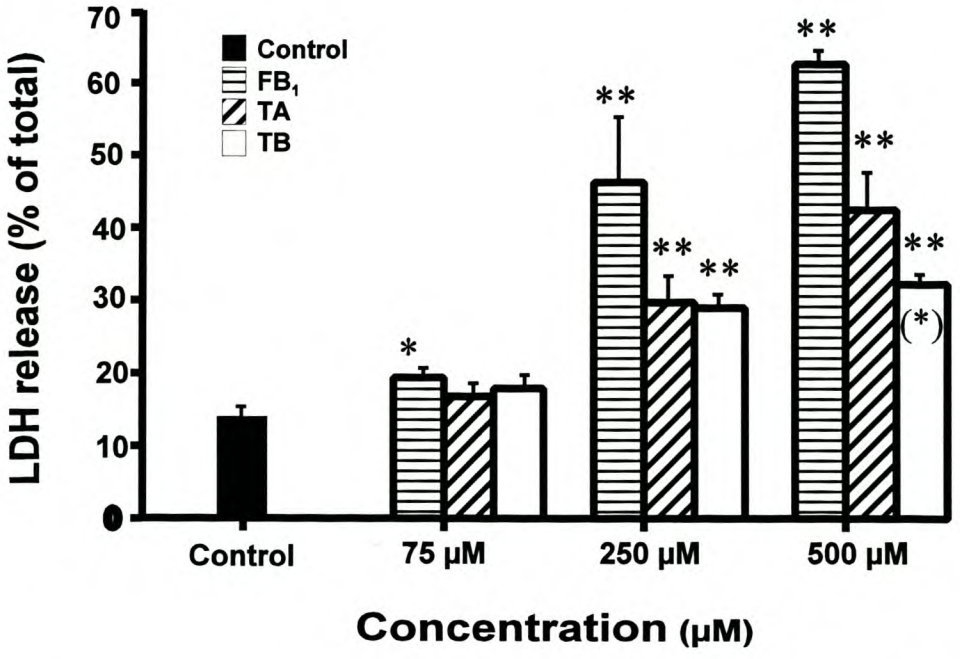

Fig. 2. *The cytotoxic effect of FB_1, TA and TB on rat primary hepatocyte cultures expressed as the amount of LDH released (% of total). Values represent means ± SD of triplicate determinations. Values differ significantly from the control treatment: *P<0.05; ** P <0.01. The cytotoxicity of TA and TB differs significantly (** P<0.01) from FB_1 at 250 and 500 µM while TB was significantly (* P<0.05 lower than TA at 500 µM.*

Comparative cytotoxicity of FB_1, TA and TB (Fig. 2)

A typical dose response effect was obtained with all the toxins. FB_1 exhibited the highest cytotoxicity at concentrations of 75 ($p<0.05$), 250 ($p<0.01$) and 500 µM ($p<0.01$) as compared to the control. TA and TB exhibited similar cytotoxicities at 250 µM ($p<0.01$) as compared to the control, while TA tended to be slightly ($p<0.05$) more toxic than TB at 500 µM.

DISCUSSION

FB_1 concentrations from 5 to 500 µM did not increase the Sa/So ratio in hepatocyte cultures significantly above that which was achieved with 1 µM FB_1 (Gelderblom *et al.*, 1995). Wang *et al.* (1991) found that the Sa concentration increased 110-fold in rat hepatocyte cultures after incubation with 1 µM FB_1 for 4 days. In the present study a slight increase in the Sa and So levels and thus the Sa/So ratio between the 0 hr and 40 hr controls may be due to normal cell function. A maximum increase in the Sa concentration was observed after 12 to 24 hr after exposure to 1μM FB_1 that represent 115- to 144-fold increase as compared to the control value. The removal of FB_1 from the incubation media, even after 12 hr, did not result in a decrease in the Sa concentration and hence in the Sa/So ratio compared to that of the cells exposed to FB_1 for 40 hr (Table 1). Therefore, the inhibition of ceramide synthase is either persistent or the Sa does not easily diffuse out of the cells (Merrill *et al.,* 1993b). It seems that the inhibition of sphingolipid biosynthesis by the fumonisins is an early event that cannot

solely be associated with the adverse biological effects induced by FB_1 in primary hepatocytes. This can be deduced from the finding that the inhibitory effect of FB_1 on the epidermal growth factor (EGF) mitogenic response in primary hepatocyte cultures is reversible upon removal of the toxin (Gelderblom *et al.*, 1995). In addition, no direct involvement of the sphingolipids, Sa or So on the EGF response in primary hepatocytes was noticed. The inhibitory effect of the EGF response in primary hepatocytes is a common property of many liver cancer promoters, including FB_1 (Gelderblom *et al.*, 1996). The present finding concerning the irreversibility of ceramide synthase inhibition further supports the hypothesis that the disruption of sphingolipid biosynthesis seems not to be a key event in the inhibition of growth stimulatory effects in primary hepatocytes. A similar type of response was noticed in LLC-PK_1 cells, a pig renal epithelial cell line, where FB_1 (35 µM) inhibited cell proliferation, as measured by protein content, and increased the Sa/So ratio over a 48-hr period (Yoo *et al.,* 1992). Those LLC-PK_1 cells, which survived FB_1 exposure, resumed normal cell growth after removal of the FB_1, indicating that the FB_1-induced inhibition of cell proliferation is also reversible in these cells.

FB_1 is not cytotoxic to primary hepatocytes when exposed for 4 days to concentrations of 1 µM (Wang *et al.*, 1991). Even at higher concentrations (50 to 250 μM), fumonisins exhibit a low to moderate cytotoxicity in primary hepatocytes as measured by LDH release (Gelderblom *et al.*, 1993). As FA_1 is less cytotoxic than FB_1 at concentrations of 125-1000 µM, it was suggested that the free amino-group plays a role in the *in vitro* cytotoxicity (Gelderblom *et al.*, 1993). On the other hand, AP_1 is known to be more

cytotoxic to primary rat hepatocytes than FB_1 (Gelderblom *et al.*, 1993). In the present study, AP_1 increased the Sa level and the Sa/So ratio to a much lesser extent than FB_1. This indicated that the tricarballylic (TCA) moieties are required for maximal inhibition of ceramide synthase. TA and TB, which are also less toxic than FB_1, significantly ($p<0.01$) increased the Sa concentration above that of FB_1 although the Sa/So ratios were very similar due to variations in the So concentrations. It would therefore, appear that a single TCA group is also sufficient for maximal ceramide synthase inhibition under the present conditions. Despite the significant ($p<0.01$) increases in Sa levels induced by FA_1 when compared to FB_1, the Sa/So ratio is of the same order due to variations in the So concentration. As compared to the presence of the TCA groups, it seems that the presence of a free amino-group is not a requisite for enzyme inhibition. Although the structural basis for the inhibition of ceramide synthase is not known, two possible modes of inhibition have been postulated (Merrill *et al.*, 1993b). The structural similarity in the head group of the toxins and the sphingoid bases (Fig. 1) allows the enzyme to recognize them as substrates or, alternatively, the tricarballylic acid moieties interact with the binding site for the fatty acid moiety. The inhibition occurring with AP_1 indicates that the former is the most probable mode, although the presence of a tricarballylic acid moiety seems to further enhance the interactions with the enzyme.

Regarding FB_1 and AP_1, the absence of the TCA moieties increased the cytotoxic effect in primary hepatocytes, possibly due to a decrease in the polarity of the molecule (Gelderblom *et al.*, 1993). In the case of TA and TB the presence of a single TCA moiety seems not to correlate with cytotoxicity as TA is more cytotoxic than TB while

both toxins are less cytotoxic than FB_1. In this regard other structural differences between the fumonisins and TA and TB also could play a contributing role. The contrasting results between cytotoxicity and the elevation of Sa levels and Sa/So ratios in primary hepatocytes, indicate that the cytotoxicity of these compounds is not solely due to inhibition of ceramide synthase and the subsequent changes in sphingoid base concentrations. The lack of direct association of cytotoxicity and the inhibition of sphingolipid biosynthesis in rat hepatocytes has previously been reported. Hepatocytes exposed to both toxic and non-toxic concentrations of FB_1 interrupt sphingolipid biosynthesis to the same extent as the concentration (1 μM) used in the present study (Gelderblom *et al.,* 1995, 1996). It would appear that the structural requirements for cytotoxicity and inhibition of ceramide synthase differ in primary hepatocytes. In contrast to this, studies on LLC-PK_1 cells have shown a direct correlation between FB_1-induced cytotoxicity and inhibition of sphingolipid biosynthesis (Yoo *et al.*, 1992). These differences may be related to the lower cytotoxicity and greater sensitivity to sphingolipid disruption by FB_1 in primary hepatocytes as compared to the LLC-PK_1 renal cells.

In vivo studies on the cancer-initiating potential of the structural analogues, AP_1, and FB_1, FB_2 and FB_3 indicated that only the fumonisin B mycotoxins exhibited activity in a short-term carcinogenesis model in rat liver (Gelderblom *et al.* 1993). The present investigation showed that differences exist in the structural requirements for the induction of cytotoxicity in primary hepatocytes and the inhibition of ceramide synthase. Future studies into the mechanism of action of the fumonisins and AAL toxins

concerning their biological effect on and role in ceramide synthase inhibition might enhance current knowledge.

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Chapter 2

The effect of a single gavage dose of fumonisin B_1 on the sphinganine and sphingosine levels in vervet monkeys

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Van der Westhuizen L., Shephard G. S. and Van Schalkwyk D. J. (1999) The effect of a single gavage dose of fumonisin B_1 on the sphinganine and sphingosine levels in vervet monkeys. *Toxicon*

ABSTRACT

This is the first report of sphinganine (Sa) and sphingosine (So) levels determined in serum and urine of vervet monkeys (*Cercopithecus aethiops*) dosed with pure fumonisin B_1 (FB_1). Initially, experimental vervet monkeys were given a single gavage dose of either 1 or 10 mg FB_1 /kg body weight. Blood and urine were sampled daily and on day seven the monkeys were sacrificed and the kidneys and livers harvested. In a subsequent experiment, other vervet monkeys were similarly dosed and blood and urine samples were collected over a 50 day period. In the high-dose monkeys the serum Sa/So ratio, as well as levels of serum cholesterol and liver function enzymes, increased during the first week after dosing and remained elevated for several weeks thereafter. The urinary Sa/So ratio and the serum renal function indicators showed a more rapid response and a correspondingly more rapid return to pre-dosing levels. In the low-dose monkeys serum Sa and the Sa/So ratio were the only parameters to increase above the control levels. The Sa/So ratio in liver and kidney tissue showed an elevation over controls in a dose-dependent manner. The serum Sa/So ratio was exclusively elevated above the control levels in the low- and high-dose monkeys and seems more relevant as a marker for fumonisin exposure than any of the other indicators.

Abbreviations: ALT = Alanine transaminase; AST = aspartate transaminase; FB_1 = fumonisin B_1; GGT = ɣ-glutamyltransferase; HPLC = high-performance liquid chromatography; LDH = lactate dehydrogenase; OPA = *o*-phthaldialdehyde; So = sphingosine; Sa = sphinganine.

INTRODUCTION

Fumonisin B_1 (FB_1) is the major mycotoxin produced by *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon), a fungus that occurs worldwide on maize (Shephard *et al.*, 1996a). FB_1 causes various syndromes in different animal species, namely leukoencephalomalacia in horses (Marasas *et al.,* 1988; Kellerman *et al.*, 1990), pulmonary oedema in pigs (Harrison *et al.,* 1990) and hepatocarcinoma in rats (Gelderblom *et al.,* 1991). High levels of fumonisins have been found in maize from the high incidence areas of oesophageal cancer in the Transkei region of the Eastern Cape province of South Africa (Rheeder *et al.*, 1992) as well as in the Cixian and Linxian counties in the Republic of China (Chu and Li, 1994). As fumonisins occur widely around the world in maize products intended for human consumption, it raises concern over the potential hazard to human health posed by these compounds (Marasas, 1997; Shephard *et al.*, 1996a; Thiel *et al.*, 1992).

Direct measurement of FB_1 in plasma as a biomarker is not feasible as it is eliminated rapidly from plasma, has a low bioavailability and lacks a major metabolite in experimental animals (Shephard *et al.*, 1998). The primary target of the fumonisins in animal cells is sphingosine (So) [sphinganine (Sa)] *N*-acyltransferase (ceramide synthase), a key enzyme in the *de novo* sphingolipid biosynthetic pathway (Wang *et al.,* 1991). The inhibition of ceramide synthase leads to an elevation of Sa levels in cells, thus resulting in an increase in the Sa/So ratio (Riley *et al.*, 1994b). An elevation in the Sa/So ratio after exposure to FB_1 has previously been observed in serum and/or urine of ponies (Wang *et al.*, 1992), pigs (Riley *et al.*, 1993), rats (Riley *et al.*, 1994b, Voss

et al., 1995, Castegnaro *et al.*, 1996, Wang *et al.*, 1999), rabbits (Gumprecht *et al.*, 1995) and horses (Goel *et al.*, 1996). Increased Sa/So ratios have also been observed in liver and/or kidney tissues of some of these experimental animals due to FB_1 exposure. In addition to these changes certain of these experimental animals had increased serum cholesterol levels and also elevations in certain or all of the serum levels of the liver function enzymes [alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyltransferase (GGT) and lactate dehydrogenase (LDH)]. FB_1 also increased the serum indicators of renal function, urea and/or creatinine, in rabbits (Gumprecht *et al.*, 1995) and rats (Gelderblom *et al.*, 1991, Lim *et al.*, 1996). As the changes in the Sa/So ratio occur before other biochemical markers of cellular injury, it has been proposed that the Sa/So ratio could be a possible biomarker for fumonisin exposure (Riley *et al.*, 1994a).

In the initial part of this study, the effect of single gavage doses of 1 and 10 mg FB_1 /kg body weight were monitored in vervet monkeys (*Cercopithecus aethiops*) for seven days, where after the primates were sacrificed. The Sa and So levels were determined daily in serum and urine of the dosed monkeys as well as in two control monkeys and, on termination of the experiment, also in liver and kidney tissues. Serum cholesterol and serum levels of certain liver function enzymes (ALT, AST, GGT and LDH) were monitored. Renal function was monitored by measuring urea and creatinine levels in the serum. As the parameters monitored in the initial study had not returned to baseline levels within seven days, a further similar study was undertaken in which monkeys were given identical doses of FB_1 and monitored until all parameters returned to normal (36 - 50 days). These monkeys were not terminated at the end of the study.

MATERIALS AND METHODS

Animals

The 10 vervet monkeys (*Cercopithecus aethiops*) used in this study were six female monkeys weighing between 2.5 and 3.6 kg and four male monkeys weighing between 4.7 and 5.7 kg. During the experimental period the monkeys were confined in individual cages with normal access to feed and water. Experimental protocols were ethically approved by the Ethics Committee for Research on Animals of the Medical Research Council, Tygerberg, South Africa.

Chemicals

FB_1 was purified as described previously by Cawood *et al.* (1991). Sa and So were obtained from Sigma Chemical Company (St. Louis, MO, USA). C_{20}-Sa was a generous gift from Prof. A. H. Merrill Jr., (Dept. of Biochemistry, Emory University, School of Medicine, Atlanta, GA, USA). All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

Experimental procedures

(i) Initial experiment

Two vervet monkeys were dosed with 1 and 10 mg FB_1 /kg body weight, respectively, in a single dose by gavage and sacrificed after seven days. Blood samples were collected by venipuncture prior to dosing and then on days 1, 2, 3, 4, 5 and 7 and liver and kidneys were harvested after the monkeys were sacrificed. Twenty-four hr urine collections were made over the period prior to drawing blood. A similar set of samples

was obtained from two undosed monkeys used as controls. Serum was obtained by allowing the blood to clot, followed by centrifugation at 1200 x *g* for 10 min at 4°C. Sa and So analyses were performed on the serum, urine, liver and kidneys.

(ii) 36- and 50-Day experiment

Two vervet monkeys per dosage level were administered a single dose of FB_1 by gavage of either 1 or 10 mg FB_1 /kg body weight. Blood samples were collected by venipuncture prior to dosing and then on days 1, 2, 3, 4, 5, 7, 10, 15, 22, 29, 36, 43 and 50 for the high-dose monkeys as well as for two control monkeys. Blood samples were collected on the same days from the low-dose monkeys, except for days 43 and 50. Twenty-four hr urine collections were made over the period prior to drawing blood.

Analytical methods

(i) Determination of Sa and So in serum, urine, liver and kidney

Sa and So levels in serum, urine, liver and kidney were determined using the method of Riley *et al.* (1994a) with minor modifications. The liver and kidney tissue were homogenized in liquid nitrogen and dissolved in 4 vol. (w/v) 0.05 M phosphate buffer (pH 7.0). The concentrations were determined by high-performance liquid chromatography (HPLC) with C_{20}- Sa as an internal standard. The lipids were extracted from either the tissue suspension, serum or urine by incubation with methanol-chloroform (2:1) (containing 0.01% butylated hydroxytoluene as an antioxidant) under nitrogen at 37°C for 1 hr. Thereafter the mixture was washed twice with alkaline water, the phases separated by centrifugation and the chloroform fraction dried under nitrogen below 40°C. The residue was hydrolysed to release the free bases by redissolving it in

0.1 M methanolic potassium hydroxide-chloroform (4:1), and incubating it at 37°C for 1 hr. After washing with alkaline water, the chloroform phase was dried under nitrogen below 40°C. The dried residues were stored at -20°C overnight. Prior to analysis, residues were redissolved in 250 µl methanol, sonicated and derivatised with 50 µl *o*-phthaldialdehyde (OPA) reagent prepared as previously described (Riley *et al.*, 1994a). A 25-75 µl aliquot was injected into the HPLC, which consisted of a Waters (Milford, MA, USA) Model 510 solvent delivery system, Waters U6K injector, Waters Radial-Pak™ cartridge packed with Nova-Pak C_{18} (4 µm, 100 x 8 mm), Autochrõm APEX Integration Chromatography Workstation and Perkin-Elmer (Norwalk, CT, USA) 650 S fluorescence detector (excitation - 335 nm and emission - 440 nm). The isocratic mobile phase of methanol/0.005 M potassium phosphate buffer, pH 7.0 (91:9) was pumped at a flow rate of 2 ml/min.

(ii) Determination of chemical pathology parameters

Serum cholesterol and serum levels of certain liver function enzymes (AST, ALT, GGT and LDH), as well as serum urea and creatinine for renal function, were measured by Technicon SMAC autoanalysis.

RESULTS

(i) Initial experiment

The concentrations of So and Sa and the Sa/So ratio in serum and urine of the monkeys sacrificed after seven days are shown in Table 1. The serum Sa and So levels

Table 1. *The mean sphingosine (So) and sphinganine (Sa) levels and the Sa/So ratio in the serum of two vervet monkeys dosed once each with FB_1 and sacrificed after 7 days.*

Time period (day)		0	1	2	3	4	5	7
Sphinganine	Control	8.4	12	5.5	5.4	11	13	13
	1 mg FB_1 /kg	25	21	40	19	39	35	46
	10 mg FB_1 /kg	28	60	144	119	267	276	279
Sphingosine	Control	10	8.7	7.6	7.6	11	14	16
	1 mg FB_1 /kg	80	56	73	20	61	48	49
	10 mg FB_1 /kg	83	105	153	99	148	171	133
Serum Ratio	Control	0.8	1.33	0.73	0.72	1.07	0.99	0.83
	1 mg FB_1 /kg	0.32	0.37	0.55	0.93	0.64	0.73	0.94
	10 mg FB_1 /kg	0.33	0.58	0.94	1.19	1.8	1.62	2.1
Urine Ratio	Control	1.14	1.22	0.9	1.15	0.87	1.66	1.51
	1 mg FB_1 /kg	0.92	1.01	1.09	0.71	0.87	1.34	0.65
	10 mg FB_1 /kg	0.85	2.28	1.78	2.78	2	1.5	0.87

in the dosed monkeys were higher than those of the control monkeys. The Sa levels in serum in the high-dose monkey increased well above that of the So from 28 to 279 nM and resulted in an increase in the ratio from 0.33 to 2.10 over the seven day period. Neither the serum nor the urinary Sa/So ratios in the low-dose monkey increased above that of the control monkeys. The serum and urinary Sa/So ratios of the high-dose monkey increased above that of the low-dose and control monkeys with the urine showing an earlier increase than the serum. However, the urinary ratio returned to its original level within the seven-day experimental period, while the serum ratios were still increasing after seven days.

The concentrations of So and Sa and the Sa/So ratio in kidney and liver of the monkeys

Table 2. *The mean sphingosine (So) and sphinganine (Sa) levels and the Sa/So ratio in the tissues of two vervet monkeys dosed once each with FB_1 and sacrificed after 7 days.*

	Sphinganine (pmol/mg protein)			Sphingosine (pmol/mg protein)			Ratio Sa/So		
Dose (FB_1 /kg)	Control	1 mg	10 mg	Control	1 mg	10 mg	Control	1 mg	10 mg
kidney[a]	6.5	42	119	15	60	58	0.43	0.71	2.05
liver[a]	4.4	20	45	16	24	23	0.28	0.83	1.97

[a] *Determined after sacrifice on day 7.*

sacrificed after seven days are shown in Table 2. The So levels in the liver of the monkeys were similar, while the levels in the kidneys of the dosed monkeys were four-fold higher than the level in the control monkeys. The Sa levels in the kidney of the low- and high-dose monkeys were six- and 18-fold higher, respectively than in the control monkeys, while in the liver the increases were four- and nine-fold, respectively. These effects led to a dose-dependent rise in the Sa/So ratio in kidney from 0.43 in the control monkeys to 0.71 and 2.05, respectively in dosed monkeys. Similarly the ratio in liver increased from 0.31 in the control to 0.83 and 1.97, respectively in the dosed monkeys. The serum cholesterol levels, the serum liver function enzymes (AST, ALT, LDH and GGT) and the serum renal indicators (urea and creatinine) all increased over the seven-day experimental period.

(ii) 36- and 50-Day experiment

The serum Sa/So ratio in these high-dose monkeys increased within the first week and only returned to control levels after four weeks (Fig. 1a). The effect of the FB_1 was most

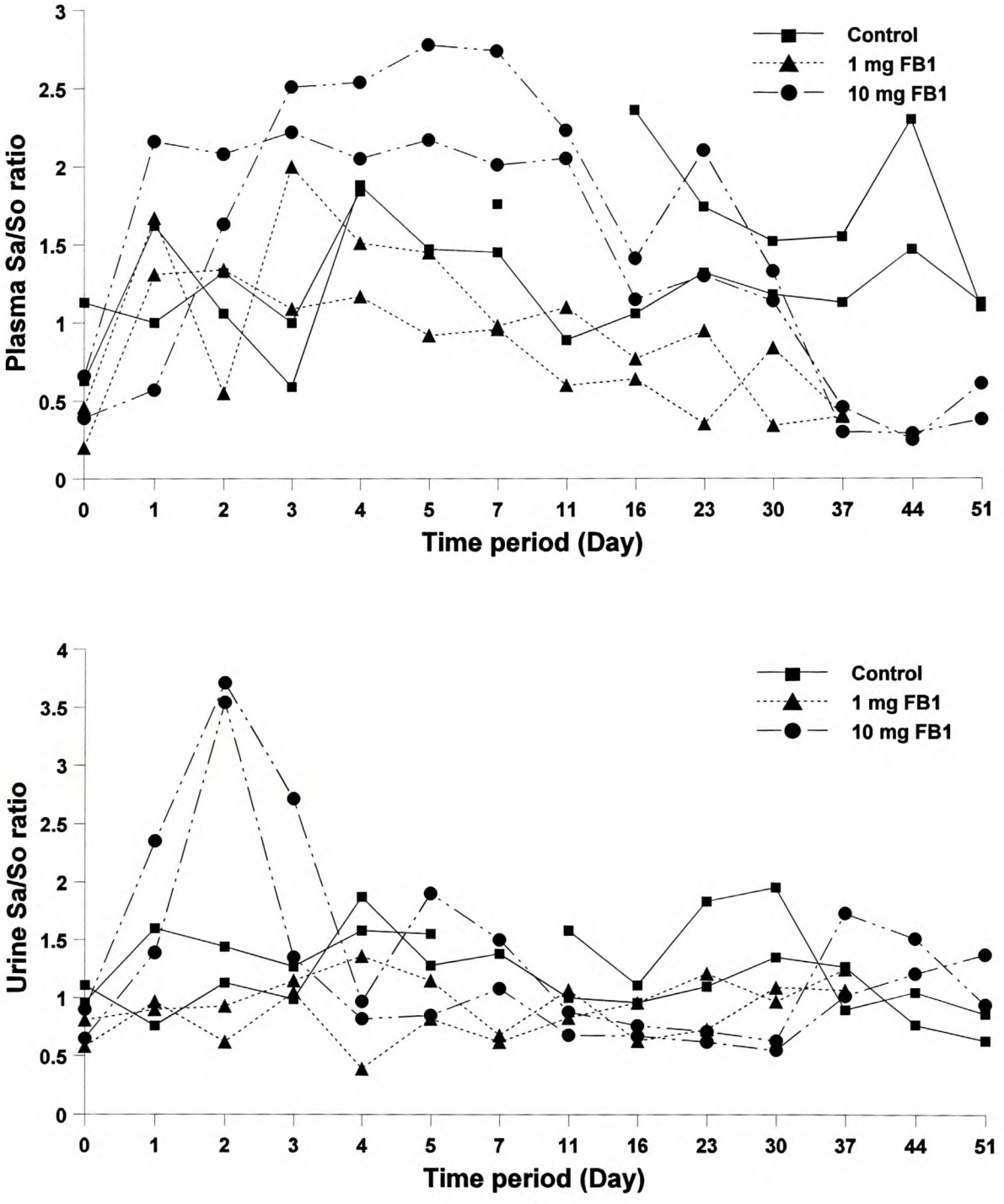

Fig. 1. *The effect of low- and high-FB_1-doses in two vervet monkeys per treatment group in the (a) serum Sa/So ratios and (b) urinary Sa/So ratios with time (days).*

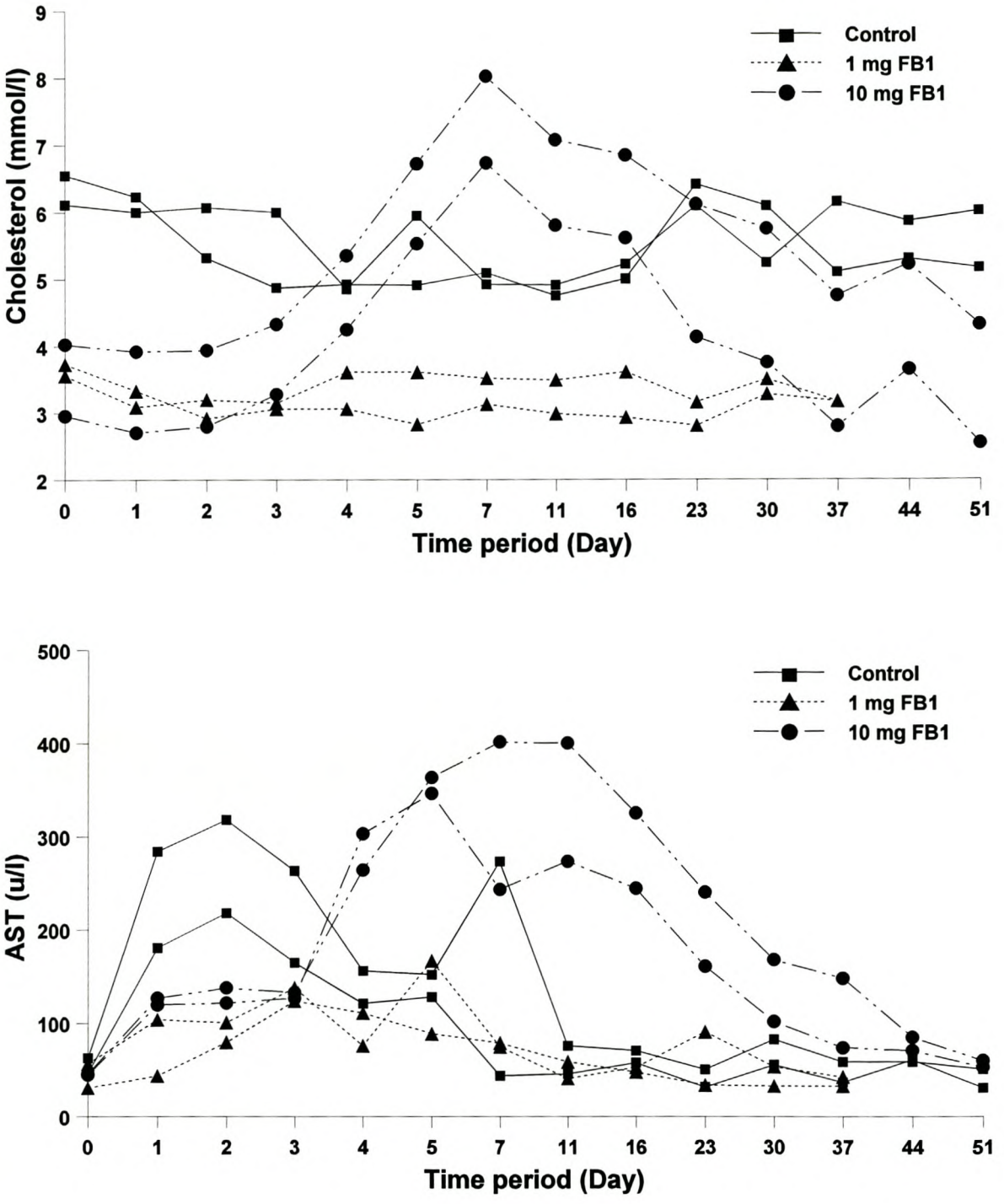

Fig. 2. *The effect of a low and high dose FB_1 in two vervet monkeys per treatment group on the (a) plasma cholesterol and (b) AST levels with time (days).*

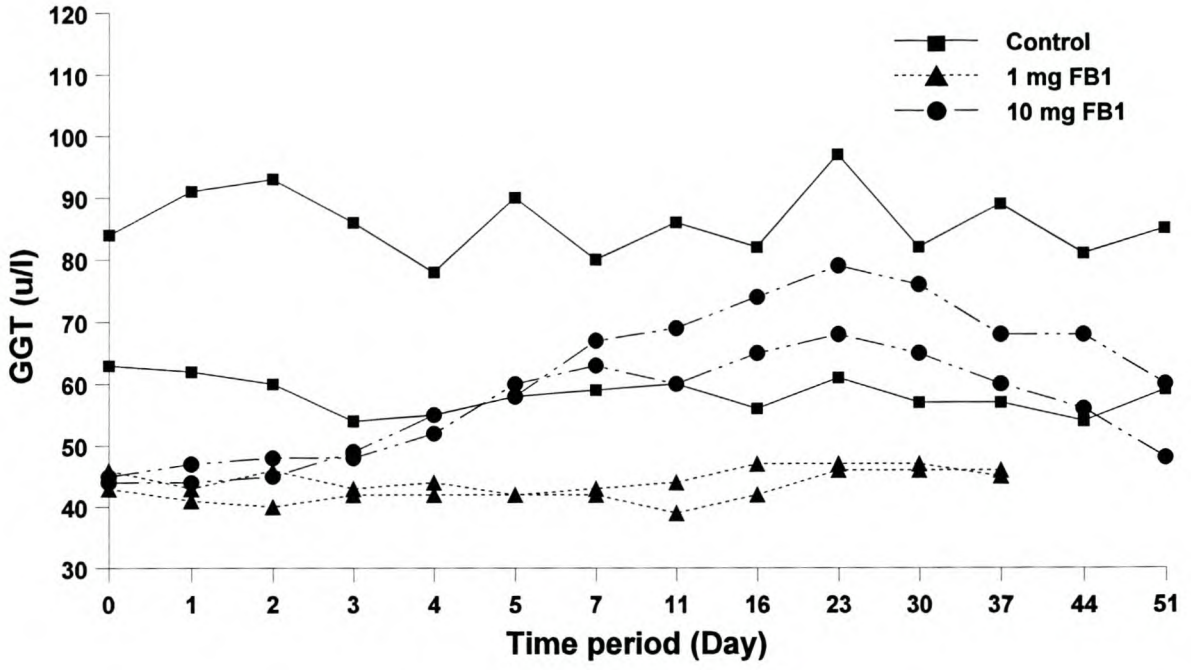

Fig. 2. *The effect of a low and high dose FB_1 in two vervet monkeys per treatment group on the (c) GGT levels with time (days).*

marked on the Sa levels in the high-dose monkeys, increasing from 30 and 37 nM prior to dosing to a maximum of 945 and 343 nM, respectively after five days. The corresponding Sa/So ratio increased from 0.39 and 0.66 prior to dosing to maxima of 2.78 and 2.17, respectively after five days. In the low-dose monkeys the serum Sa/So ratio increased from 0.46 prior to dosing to 2.00 after three days and 0.20 prior to dosing to 1.34 after two days. The urinary Sa/So (Fig. 1b) increased rapidly in the high-dose monkeys, peaked after two days at 3.54 and 3.71 and rapidly declined thereafter to pre-dosing levels after four days. The urinary Sa/So ratios in the low-dose monkeys did not differ significantly ($p>0.05$) from the control monkeys.

In the high-dose monkeys serum cholesterol (Fig. 2a), AST (Fig. 2b), ALT and LDH

(data not shown, but similar to AST) levels increased to a maximum after five to seven days. The serum cholesterol and AST levels remained elevated for a longer period than the ALT and LDH levels and then declined slowly to control levels over a six week period. AST increased nine-fold to a maximum level after seven days and LDH increased four-fold to a maximum level after five days. The GGT levels in the high-dose monkeys increased immediately, remained elevated for several weeks and only started to decline towards the end of the study period (Fig. 2c). The serum creatinine levels (data not shown), and the urea levels (data not shown) to a lesser extent, increased in a similar rapid manner to the urinary Sa/So ratio in the high-dose monkeys and returned to pre-dosing levels after seven days. In the low-dose monkeys, none of the chemical pathology parameters were elevated significantly ($p>0.05$) above those of the controls.

DISCUSSION

It is by now well established that ingestion of FB_1 leads to elevation of the Sa/So ratio in the serum, urine and tissues of several animal species due to the inhibition of the key sphingolipid biosynthetic enzyme, ceramide synthase. In the present study, single gavage doses of FB_1 produced a number of toxicodynamic responses in vervet monkeys. The initial experiment, which was terminated after seven days in order to obtain liver and kidney tissue, indicated that the disruption of sphingolipid metabolism, as evidenced by elevated serum Sa/So ratios, showed no sign of abatement up to seven days after the single gavage dose. In addition, key chemical pathological

parameters of liver damage and renal function also showed no abatement during this period. The determination of Sa/So ratio in the liver and kidney tissues indicated that a dose-dependent effect on sphingolipid metabolism occurred in both organs. Hence both liver and kidney appear to be target organs for FB_1 in vervet monkeys. FB_1 has previously been shown to be hepatotoxic in rats (Gelderblom *et al.*, 1996, Voss *et al.*, 1993), swine (Gumprecht *et al.*, 1998) and ponies (Ross *et al.*, 1993) and nephrotoxic in rabbits (Gumprecht *et al.*, 1995) and rats (Riley *et al.*, 1994b, Voss *et al.*, 1993).

The second study was designed to follow all parameters monitored in the monkeys from the time of dosing, until they returned to levels similar to those measured prior to dosing. The results clearly indicated that Sa was the main sphingoid base elevated by consumption of fumonisins and changes in So levels were secondary, thus leading to the elevation in the Sa/So ratio. This primary effect on Sa is consistent with the sphingolipid *de novo* biosynthetic pathway in which Sa is the base acylated by ceramide synthase to form dihydroceramide prior to conversion to ceramide by the formation of the double bond (Merrill *et al.*, 1997). Hence any inhibition of the ceramide synthase enzyme leads to accumulation of Sa. So, by contrast, is a product of sphingolipid turnover and, if not re-acylated to ceramide, is subjected to further catabolic metabolism (Rother *et al.*, 1992).

Toxicokinetic studies of FB_1 in vervet monkeys have shown that FB_1 has a low bioavailability and a short elimination half-life (Shephard *et al.*, 1994). Indeed, serum levels measured after a gavage dose, peaked within a couple of hours of the dose,

falling rapidly to below detection levels (Shephard *et al.*, 1995). Despite this low bioavailability and rapid elimination, the biochemical toxicodynamic effects of a single dose show sustained changes in various parameters over a much longer period. The serum Sa/So ratio is significantly ($p<0.05$) increased over their respective levels prior to dosing within the first week of the study period in low- and high-dose monkeys. Urinary levels of the ratio showed a more rapid response and a correspondingly more rapid return to pre-dosing levels. It is clear that although FB_1 may be rapidly eliminated, the biochemical changes that it induces are of an extended nature such that a single large exposure can still have measurable effects many weeks later. The free sphingoid bases in urine arise from the cellular content of urine (Riley *et al.*, 1994b) and hence it appears that despite the tissue showing elevated Sa/So ratio, the ratio measured in the cells contained in urine had returned to initial values after seven days. It is uncertain from which particular tissues the free sphingoid bases in serum arise as most, if not all, tissues appear to have the capacity for sphingoid base biosynthesis (Merrill *et al.*, 1993). As assessed by the sphingoid bases within the cellular content of urine, it appears that the kidney recovers quicker from the toxic insult of FB_1, while the effect on other tissues such as liver is of longer duration. The observed differences between the duration of response in urine and serum are a reflection of differing cellular responses. Recent studies with primary rat hepatocytes in culture have shown that the elevation of the Sa/So ratio persists after removal of FB_1 from the culture medium (Van der Westhuizen *et al.*, 1998), whereas previous studies in cultured LLC-PK_1 pig kidney cells have shown the effects of FB_1 on growth arrest and morphology to be readily reversed with removal of the toxin (Yoo *et al.*, 1992).

The chemical pathology parameters monitored for liver damage and renal function were only affected at the high dose peaking between five and seven days after the high dose, corresponding also to the maximum elevation of the Sa/So ratio in serum. The serum cholesterol increased within the first week and declined over the next six weeks of the study period. It has previously been reported that cholesterol accumulated in plasma but not in liver tissue of monkeys receiving *F. verticillioides* culture material in their diet (Fincham *et al.,* 1992). This suggests that the atherogenic effect of the culture material was due to a specific problem at the hepatocyte membrane leading to a reduced rate of plasma clearance (Fincham *et al.,* 1992). The leakage of ALT, AST, LDH and GGT into the serum is indicative of hepatotoxicity. As AST is abundant in most tissues and ALT is more specific to the liver (Aranda-Michel and Sherman, 1998), the sustained elevation of the AST levels over that of the ALT levels in the high-dose monkeys indicates that tissues other than the liver are also likely to be affected. Although GGT is a very sensitive indicator of liver injury, it is not specific to the liver (Theal and Scott, 1996) and therefore the sustained elevation of the GGT levels, together with that of AST, also indicates that other tissues besides the liver may also be affected. Likewise LDH is a indicator of liver injury, but it is less specific than AST and ALT (Johnston, 1999). Serum creatinine and urea, the renal function indicators, had the same rapid transient response as the urinary Sa/So ratio in the high dose-monkeys, indicating nephrotoxicity (Loeb, 1998). It would thus seem that FB_1 has an immediate short term effect in kidneys at the higher dose, in contrast to the sustained effect in the liver and possibly other tissues.

FB_1 increased the serum Sa/So ratio at the low dose, while none of the other serum indicators were increased above the control levels. It would thus seem that the serum Sa/So ratio is more sensitive to the low dose effects of FB_1 than the other serum indicators, while the other serum indicators showed a sustained elevation at the high dose. In this study the effect of a single large FB_1 dose could still be measured in the serum Sa/So ratio many weeks later. However, urinary levels of the ratio showed a more rapid response and a correspondingly more rapid return to pre-dosing levels. It would thus seem that the serum Sa/So ratio is more relevant as a marker for fumonisin exposure, which confirms the findings of a previous study which also indicated that changes in the serum ratio may be more sensitive than changes in the urinary ratio in vervet monkeys receiving fumonisin-contaminated feed over an extended period (Shephard *et al.*, 1996b).

This study showed the effect of a single low and high dose of FB_1 on the Sa and So profiles in serum and urine of vervet monkeys. Humans consuming maize are exposed to low repeated doses of the different fumonisins and it would therefore be relevant to study the Sa and So profiles of non-human primates exposed to the other fumonisins as well as repeated low doses of FB_1.

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Chapter 3

The effect of repeated gavage doses of fumonisin B_1 and a single gavage dose of fumonisin B_2 on the sphinganine and sphingosine levels in vervet monkeys

In preparation:

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ABSTRACT

The disruption in sphinganine (Sa) and sphingosine (So) levels in plasma and urine of vervet monkeys (*Cercopithecus aethiops*) was measured following repeated doses of 1 mg fumonisin B_1 (FB_1) /kg body weight 3 times /week or a single gavage dose of either 1 or 10 mg fumonisin B_2 (FB_2) /kg body weight. Blood and urine were collected over a 51-day period. After 30 days of repeated FB_1 doses, the plasma Sa/So ratios were increased 5-fold above those of the control monkeys and then declined slowly to double the value in controls after 51 days. The plasma Sa/So ratios in the high-FB_2-dose monkeys increased 4-fold over the levels in controls after seven days and then returned slowly to baseline levels after 51 days. In the low-dose monkeys the plasma Sa/So ratio was the only parameter that increased above the control levels. In the monkeys gavaged with repeated FB_1 doses aspartate transaminase (AST), γ-glutamyl-transferase and lactate dehydrogenase increased above the control levels after 11 days and reached maximum levels after 44 days. In the high-FB_2-dose monkeys AST and alanine transaminase peaked on day four with 13- and 12-fold increases, respectively. This study demonstrated that similar to FB_1, a single dose of FB_2 can cause disruption of sphingoid metabolism in vervet monkeys and that repeated low doses of FB_1 can cause more severe and sustained disruption of sphingoid metabolism than either a low or high single FB_1 dose.

Abbreviations: ALT = Alanine transaminase; AST = aspartate transaminase; FB_1 =

fumonisin B_1; FB_2 = fumonisin B_2; FB_3 = fumonisin B_3; GGT = γ-glutamyl transferase; HPLC = high-performance liquid chromatography; LDH = lactate dehydrogenase; OPA = *o*-phthaldialdehyde; So = sphingosine; Sa = sphinganine.

INTRODUCTION

Fumonisins are a group of food-borne mycotoxins produced as secondary metabolites by *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon) (Gelderblom *et al.*, 1988) of which fumonisin B_1 (FB_1) and B_2 (FB_2) (see Fig. 1 for structures) are the major naturally occurring analogues. This seed-borne fungus occurs world-wide on maize intended for human and animal consumption (Shephard *et al.*,1996a). Maize contaminated with fumonisin is of concern as it causes various animal diseases (Kellerman *et al.*, 1990; Harrison *et al.,* 1990) and carcinogenesis in rats (Gelderblom *et al.,* 1991). High levels of fumonisins have been found in maize from areas where high incidences of oesophageal cancer occur (Rheeder *et al.*,1992; Chu and Li, 1994).

Sphinganine (Sa) and sphingosine (So), sphingoid bases of sphingolipids, usually occur at very low levels in animal cells (Riley *et al.*, 1993) as a precursor of complex sphingolipids in the *de novo* sphingolipid biosynthetic pathway. Sa *N*-acyltransferase (ceramide synthase) converts Sa to ceramide, while So is a turnover product of ceramide and the other complex sphingolipids (Rother *et al.*, 1992). So can undergo further catabolism or be re-acylated by ceramide synthase back to ceramide (Merril *et*

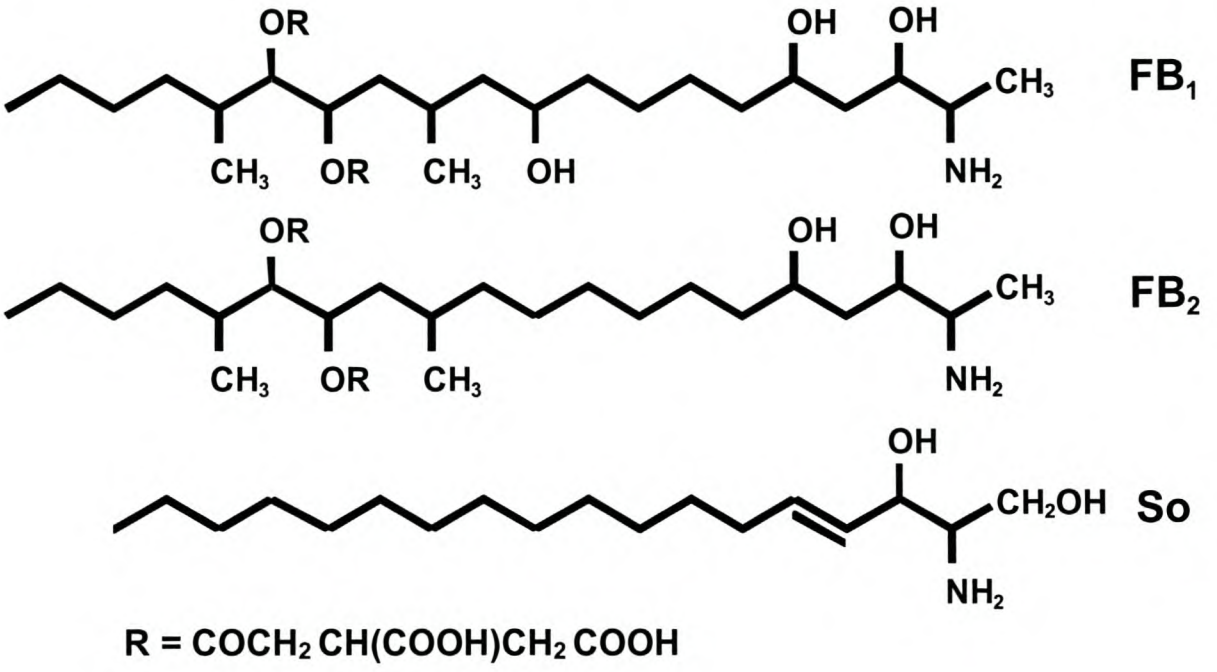

Fig. 1. *The chemical structures of FB_1, FB_2 and sphingosine.*

al., 1993). FB_1 and FB_2 inhibit ceramide synthase in rat hepatocytes (Wang *et al.*, 1991) and in rat liver slices (Norred *et al.*, 1997) and cause the accumulation of Sa, and So to a lesser extent, in cells. The inhibition of ceramide synthase is specific to fumonisins and fumonisin-like toxins as other toxins did not increase the Sa/So ratio in rat liver slices (Norred *et al.*, 1997). It has been proposed that fumonisins interact with both the binding site for Sa and the site for the fatty acyl-CoAs of ceramide synthase (Merrill *et al.*, 1993). As this disruption in the sphingolipid pathway occurs before other indicators of cellular injury, it has been proposed as a biomarker of fumonisin exposure (Wang *et al.*, 1992, Riley *et al.*, 1994b). Ponies fed diets contaminated with FB_2 had increased liver and kidney sphingoid bases, but decreased complex sphingolipid levels in these tissues (Wang *et al.*, 1992, Riley *et al.*, 1997). The disruption in the sphingolipid

pathway thus not only affects the Sa/So ratio, but also the levels of the other complex sphingolipids as has also been shown in LLC-PK_1 cells (Yoo et al., 1996). As sphingoid bases and ceramide play important roles in cell cycle progression and apoptosis (Merrill *et al.*, 1997), the disruption of the sphingolipid pathway caused by fumonisin inhibition can affect carcinogenesis (Ciacci-Zanella *et al.*, 1998).

As FB_1 is the most abundant of the fumonisins, most of the toxicological and biochemical studies have focussed on this analogue. Nevertheless, FB_2 occurs at significant levels in contaminated maize (Shephard *et al.*, 1996a) and has been shown to possess similar toxicological properties to FB_1, such as liver cancer initiation in rats (Gelderblom *et al.*, 1993), cytotoxicity to various cell lines (Abbas *et al.*, 1993; Gelderblom *et al.*, 1993; Mirocha *et al.*, 1992; Yoo *et al.*, 1992), and phytoxicity to maize and tomato plants (Lamprecht *et al.*, 1994). Like FB_1, it causes leukoencephalomalacia in horses (Ross *et al.*, 1994; Riley *et al.*, 1997) and is an inhibitor of ceramide synthase in rat lever microsomes (Wang *et al.*, 1991), rat liver slices (Norred *et al.*, 1997), primary rat hepatocytes (Van der Westhuizen *et al.*, 1998) and *in vivo* in rats (Voss *et al.*, 1998) and ponies (Riley *et al.*, 1997). The toxicokinetics of FB_2 in rats and vervet monkeys are similar to FB_1 in that it shows rapid elimination from plasma, mainly via biliary excretion and exhibits a very low bioavailability (Shephard *et al.*, 1995; Shephard and Snijman, 1999). The less polar nature of FB_2 causes it to show less urinary excretion than its more polar analogue, FB_1.

In a long term study of vervet monkeys, consumption of feed contaminated with

fumonisin-containing *F. verticillioides* culture material has been shown to lead to permanent elevation of the serum and urinary Sa/So ratios as monitored over a 60 week period within the study (Shephard *et al.*, 1996b). In a more recent study in vervet monkeys, a single high gavage dose (10 mg/kg body weight) of purified FB_1 also caused a transient increase in the serum and urinary Sa/So ratios, while a low gavage dose (1 mg/kg) only increased the serum Sa/So ratios (Van der Westhuizen et al., 1999). The effect of fumonisins on the Sa/So ratios in serum and/or urine and/or various tissues (liver, kidney, lung, brain and muscle) has been investigated in various other animal species: repeated doses of FB_1 in rats, mice and rabbits (Castegnaro *et al.*, 1996; 1998; Gumprecht *et al.*, 1995; Laborde *et al.*, 1997); FB_1 in feed of rats, catfish, angora goats, turkey poults, rainbow trout, pigs and ponies (Castegnaro *et al.*, 1996; Gelderblom *et al.*, 1996b; 1996c; 1997; Goel *et al.*, 1994; Gurung *et al.*, 1998; Ledoux *et al.*, 1996; Meredith *et al.*, 1998; Reddy *et al.*, 1995; 1996; Rotter *et al.*, 1996; Voss *et al.*, 1995; 1996; Wang *et al.*, 1992; 1999; Weibking *et al.,* 1994); FB_1 and FB_2 in feed of pigs and rats (Haschek *et al.*, 1992; Riley *et al.*, 1993; 1994a); FB_1, FB_2 and fumonisin B_3 (FB_3) in feed of mink (Morgan *et al.*, 1997; Restum *et al.*, 1995) and FB_2 or FB_3 in feed of ponies and rats (Riley *et al.*, 1997; Voss *et al.*, 1998).

Animals exposed to fumonisins had increased serum cholesterol levels and/or increases in certain or all of the serum levels of liver function enzymes [alanine transaminase (ALT), aspartate transaminase (AST), γ-glutamyl transferase (GGT) and lactate dehydrogenase (LDH)] and/or increased serum indicators of renal function, urea and/or creatinine: in turkeys, rats, mice, lambs, broiler chicks, vervet monkeys, rabbits,

angora goats, pigs, calves, mink, cows and ponies (Bermudez *et al.,* 1997; Bondy *et al.,* 1995; 1996; 1997; 1998; Edrington *et al.,* 1995; Espada *et al.,* 1994; Fincham *et al.,* 1992; Gelderblom *et al.*, 1991; 1996c; 1997; Gross *et al.*, 1994; Gumprecht *et al.,* 1995; Gurung *et al.,* 1998; Harvey *et al.*, 1995; Haschek *et al.*, 1992; Hendrich *et al.*, 1993; Javed *et al.*, 1995; Kubena *et al.,* 1995; 1997a; 1997b; Ledoux *et al.*, 1992; Lim *et al.,* 1996; Motelin *et al.,* 1994; Osweiler *et al.*, 1993; Reddy *et al.*, 1995; 1996; Restum *et al.*, 1995; Richard *et al.,* 1996; Rotter *et al.*, 1996; 1997; Smith and Thakur, 1996; Suzuki *et al.*, 1995; Voss *et al.,* 1990; 1993; 1995; 1998; Wang *et al.*, 1992; Weibking *et al.,* 1993).

Although a previous study (Van der Westhuizen *et al.*, 1999) has addressed the effect of single doses of FB_1 on sphingoid metabolism in vervet monkeys, animals and humans are generally exposed to both FB_1 and FB_2, frequently as repeated exposure to the same contaminated feed or food source. This study was designed to investigate the effect of repeated exposure to a low FB_1 dose and also the effects of single exposure to pure FB_2. This paper reports the effects on the levels of Sa and So of repeated doses (three times /week) at 1 mg FB_1 /kg body weight or single gavage doses of either 1 or 10 mg FB_2 /kg body weight in two monkeys *(Cercopithecus aethiops*) per dosage group over a 51 day period. In addition plasma cholesterol and plasma levels of certain liver function enzymes (ALT, AST, GGT and LDH) were monitored. Renal function was monitored by measuring the plasma urea and creatinine levels.

MATERIALS AND METHODS

Animals

The eight vervet monkeys (*Cercopithecus aethiops*) used in this study were juvenile female monkeys weighing between 2.44 and 3.10 kg. During the experimental period the monkeys were confined in individual cages with normal access to feed and water. Experimental protocols were ethically approved by the Ethics Committee for Research on Animals of the Medical Research Council, Tygerberg, South Africa.

Chemicals

FB_1 and FB_2 were purified as described previously by Cawood *et al.* (1991). Sa and So were obtained from Sigma Chemical Company (St. Louis, MO, USA). C_{20}-Sa was a generous gift from Prof. A. H. Merrill Jr, (Department of Biochemistry, Emory University, School of Medicine, Atlanta, GA, USA). All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

Experimental procedures

Two vervet monkeys per treatment group were dosed by gavage with 1 mg FB_1 /kg body weight three times /week or with either 1 or 10 mg FB_2 /kg body weight, respectively, in a single gavage dose. Blood samples were collected by venipuncture from experimental monkeys and from 2 control monkeys prior to dosing and then on days 1, 2, 3, 4, 5, 7, 11, 16, 23, 30, 37, 44 and 51. Plasma was obtained by centrifugation at 1200 x *g* for 10 min at 4°C and stored at -20°C until analysed. Urine

collections were made over the 24-hr period preceding each blood sample collection.

Analytical methods

(i) Determination of Sa and So in plasma and urine

Sa and So levels in plasma and urine were determined using the method of Castegnaro *et al.* (1998). In short 0.8 % potassium chloride and 1 M potassium hydroxide were added to plasma or urine and extracted with ethyl acetate by shaking gently for 20 minutes. The phases were separated by centrifugation and the ethyl acetate was dried down under nitrogen gas below 37°C. The sphingoid bases were quantified by HPLC with C_{20}- Sa as an internal standard. Prior to analysis, residues were redissolved in methanol and derivatised with *o*-phthaldialdehyde (OPA) reagent. An aliquot was injected into the high-performance liquid chromatograph (HPLC), which consisted of a Waters (Milford, MA, USA) Model 510 solvent delivery system, Waters U6K injector, Phenomenex Luna 3µ C_{18} column (75 x 4.6 mm I.D.), Autochrõm APEX Integration Chromatography Workstation and Waters Fluorescence 474 detector (excitation - 335 nm and emission - 440 nm). The isocratic mobile phase of methanol / 0.07 M potassium phosphate buffer (90:10) was pumped at a flow rate of 1 ml /min.

(ii) Determination of chemical pathology parameters

Plasma cholesterol and plasma levels of certain liver function enzymes (AST, ALT, GGT and LDH), as well as plasma indicators of renal function, urea and creatinine, were measured by Technicon SMAC autoanalysis.

RESULTS

Repeated FB_1 dose: 1 mg three times /week

The plasma Sa/So ratios in the two monkeys showed a marked increase over predose levels 4 days after the first dose (and 2 days after the second). Thereafter, the ratio increased to maxima of 3.78 and 4.76 after 30 days, and subsequently declined to 2.40 and 2.66 at the end of the study period (Fig. 2a). Urinary Sa/So ratios in controls varied between 0.63 and 1.95 over the 51-day period during which time the dosed monkeys showed no consistent pattern of Sa/So ratio elevation with the ratio remaining almost exclusively within the range of controls (data not shown). The plasma cholesterol (Fig.2b), AST (Fig.2c) and GGT (Fig. 2d) levels increased above the control levels within 7 to 11 days after dosing and while the cholesterol and AST levels remained elevated above the control levels, the GGT levels continued to increase over the remainder of the study period. Although the plasma ALT and LDH levels (data not shown) increased above the levels of the control monkeys within the first couple of days and remained elevated throughout the study period, they only increased marginally above the levels prior to dosing. Plasma urea and creatinine showed no consistent trend during the study period.

Single FB_2 dose: (i) low dose (1 mg)

The plasma Sa/So ratios increased after 3 days, peaked after 7 days with 4-fold increases over the levels prior to dosing (maximum ratios of 2.17 and 3.09) and declined to control levels after 37 to 44 days (Fig. 3a). The urinary Sa/So ratios and

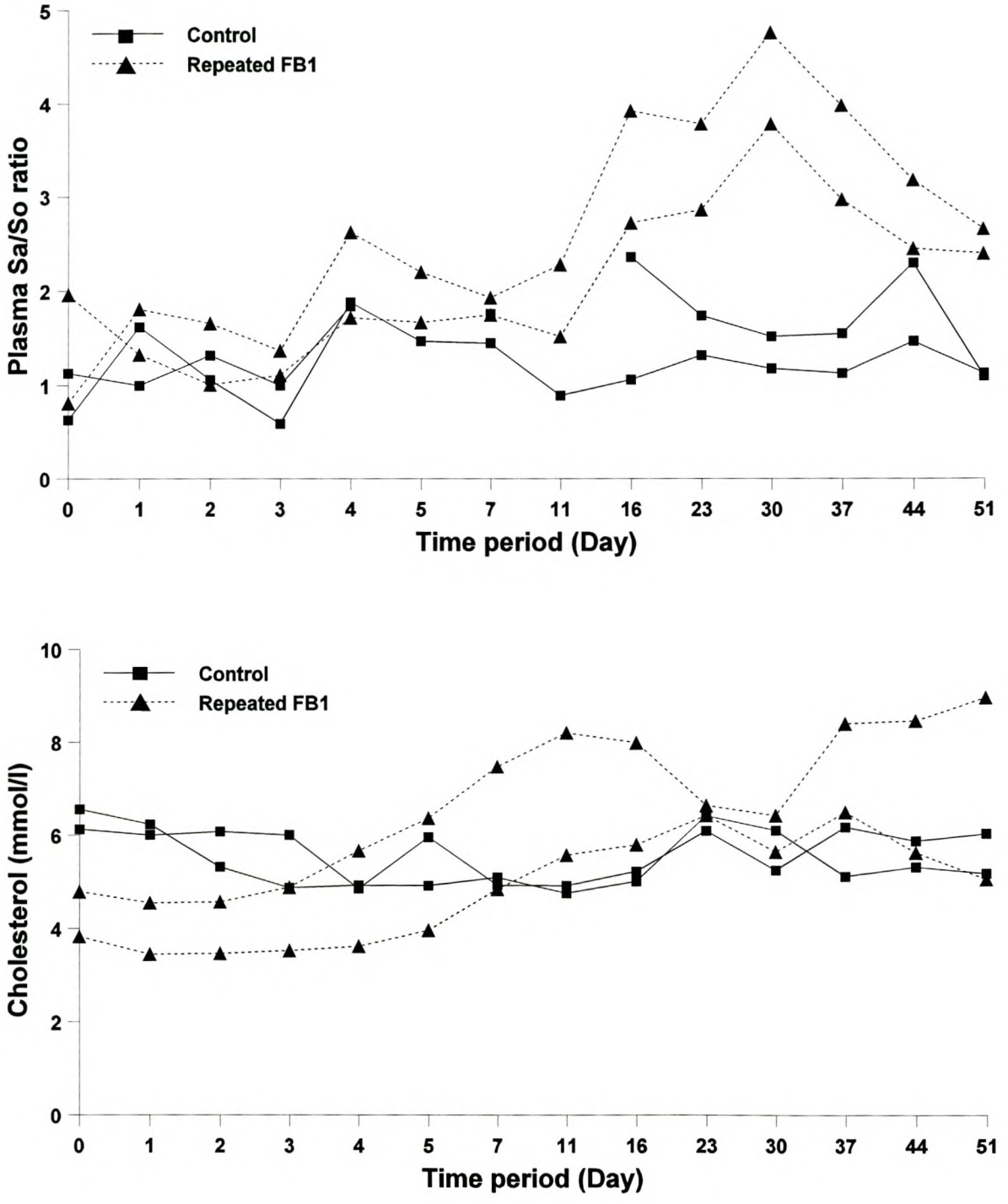

Fig. 2. *The effect of repeated doses of 1 mg FB_1 on the plasma (a) Sa/So ratio and (b) cholesterol with time (days) in two vervet monkeys per treatment group.*

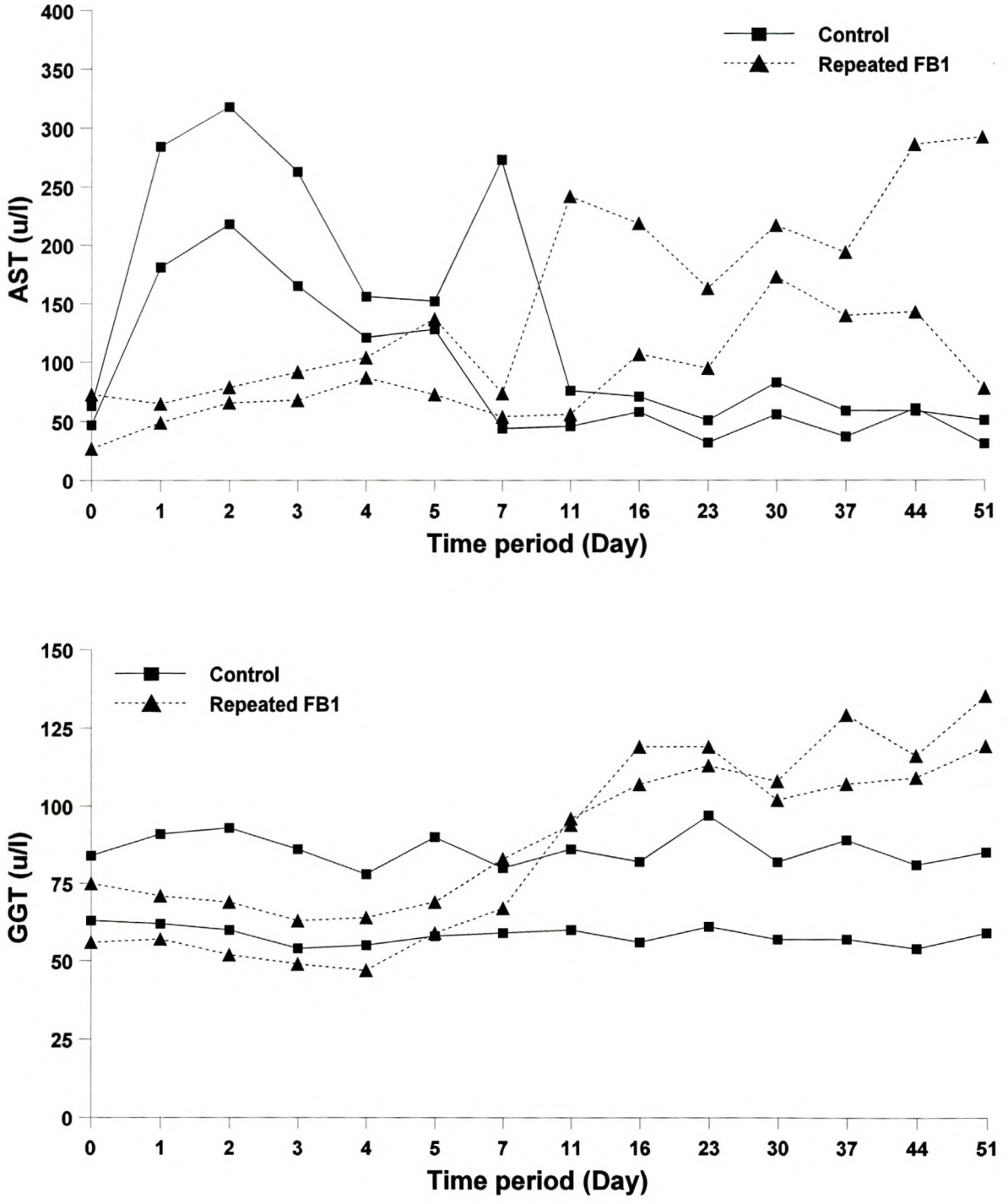

Fig. 2. *The effect of repeated doses of 1 mg FB_1 on the plasma (c) AST and (d) GGT with time (days) in two vervet monkeys per treatment group.*

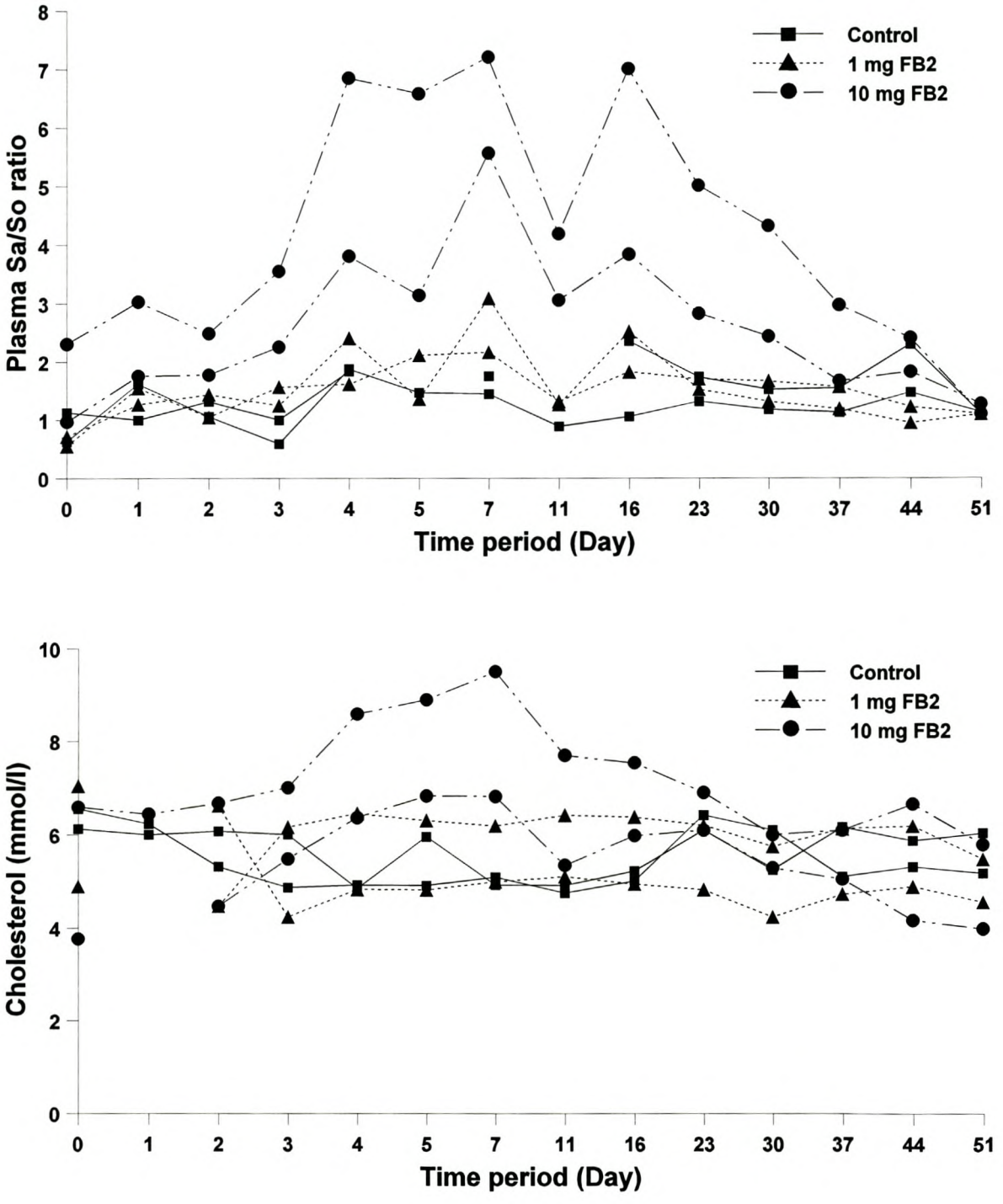

Fig. 3. *The effect of a single gavage dose of FB_2 on the plasma (a) Sa/So ratio and (b) cholesterol with time (days) in two vervet monkeys per treatment group.*

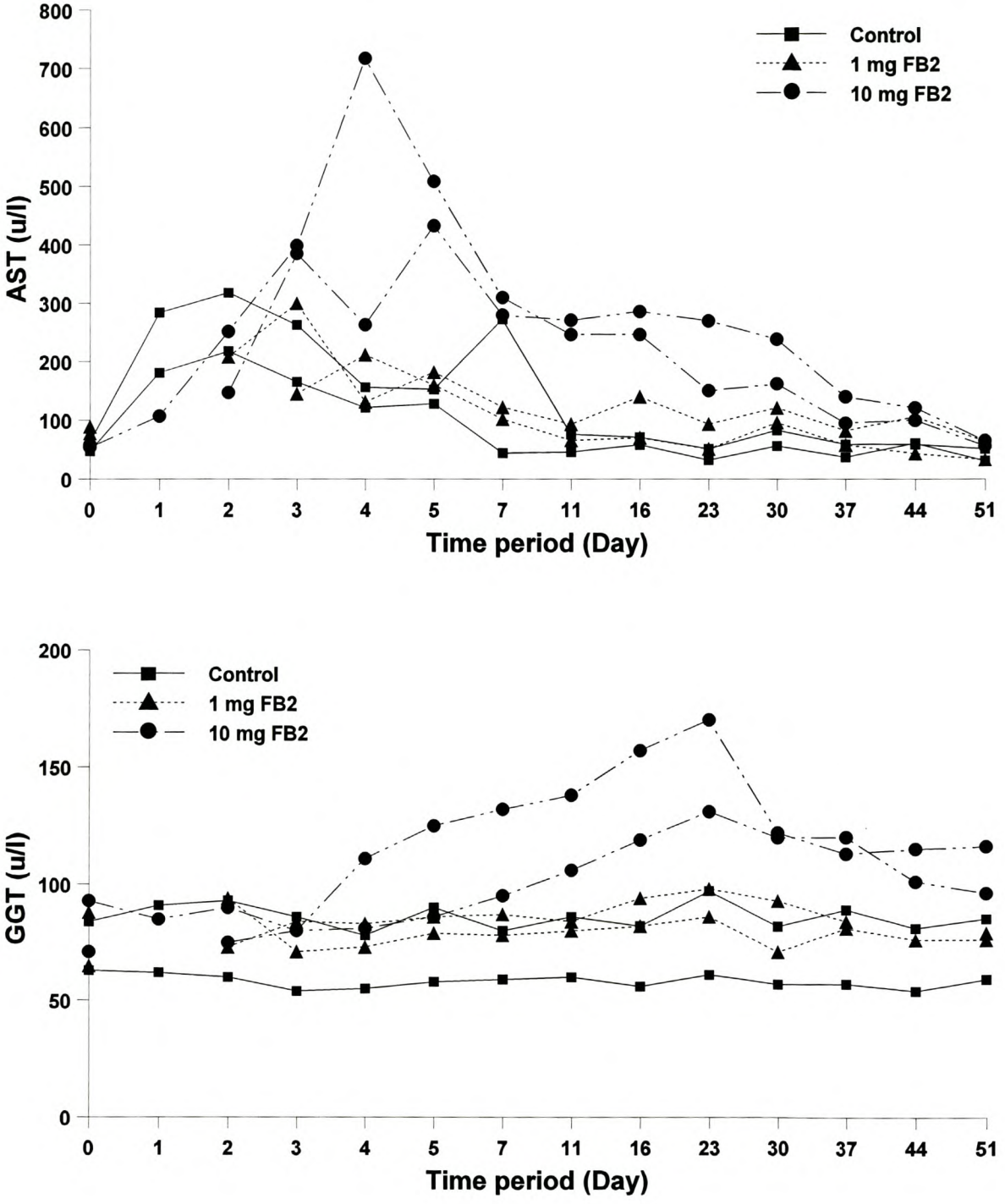

Fig. 3. *The effect of a single gavage dose of FB_2 on the plasma (c) AST and (d) GGT with time (days) in two vervet monkeys per treatment group.*

other biochemical plasma parameters did not increase above the levels of the control monkeys (data not shown).

Single FB_2 dose: (ii) high dose (10 mg)

The plasma Sa/So ratios increased after 3 days, peaked after 7 days with 3- and 6-fold increases (maximum ratios of 5.57 and 7.21) over the levels prior to dosing and declined to control levels after 51 days (Fig. 3a). The urinary Sa/So ratios did not increase above the levels of the control monkeys. The plasma cholesterol (Fig. 3b), AST (Fig. 3c) and LDH levels (data not shown) increased rapidly and peaked between 4 and 7 days with maximum elevations of 2-, 13- and 7-fold, respectively, above the levels prior to dosing. Thereafter, the levels declined steadily and returned to predosing levels by the end of the study period. The plasma ALT levels increased rapidly, peaked after 3 and 4 days respectively with 10- and 13-fold increases above predosing levels and declined rapidly to these levels after 16 days (data not shown). The plasma GGT levels increased steadily and peaked after 23 days with 2-fold increases over the initial levels, where after the levels declined steadily but remained above predosing levels at the end of the study period (Fig. 3d). The plasma creatinine and urea levels did not increase above the control levels (data not shown).

DISCUSSION

This study shows that repeated FB_1 doses of 1 mg/kg body weight in vervet monkeys

resulted in the sustained elevation of plasma Sa/So ratios and blood biochemistry parameters (liver enzymes and cholesterol). The previous study, in which single 1 and 10 mg/kg doses of FB_1 were administered to vervet monkeys, demonstrated that the toxicological and biochemical effects of FB_1 progressively appear as increases in serum Sa/So ratios and elevations in plasma cholesterol and liver function enzymes some days after the oral dose, peak at around 7 days after the dose and then gradually decline to normal levels over an extended period of weeks depending on the size of the initial dose (Van der Westhuizen *et al.*, 1999). Toxicokinetic studies have demonstrated that this single FB_1 dose is excreted, mainly in feces, over the period of a week following the dose (Shephard *et al.*, 1994). As in the single dose experiment at 1 mg/kg, the Sa/So ratio in plasma in this study increased within days of the first dose. The effect of the repeated dose was to cause the ratio to increase in steps and reach a maximum (3.78 and 4.76) after 30 days. The levels measured at the end of the experimental period are similar to those recorded in monkeys exposed in the long term to fumonisin-containing *F. verticillioides* culture material (Shephard *et al.*, 1996b). The decline from the maximum at 30 days to these levels at 51 days suggests the possibility of some adaptive response in the monkey. Accumulated sphingoid bases are subject to further metabolism such as the formation of sphinganine phosphate and *N*-acetyl-sphinganine (Merrill, A. H. Jr, Personal communication). The lack of a clear elevation in urinary Sa/So ratio after 51 days of multiple exposure indicates that in monkeys, the plasma ratio is more sensitive than urinary changes. This result reflects the outcome of the single dose experiments in which the urinary Sa/So ratios were only affected in the high-dose monkeys. In this regard, no indications were found in plasma of renal toxicity from the repeated doses, as urea and creatinine levels failed to rise to abnormal levels.

Although the liver enzymes and plasma cholesterol failed to respond to a single 1 mg/kg FB_1 dose, all these parameters showed sustained elevations from the repeated doses. However, these changes are not specific to fumonisins and are merely indicators of liver injury and the atherogenic response caused by fumonisins in monkeys (Fincham *et al.*, 1992). The selection of the 1 mg/kg level for this multiple exposure experiment with FB_1 was based on results of the previous single dose experiments in which marginal effects were seen at this level as compared to responses to the high dose (10 mg/kg). Based on a dose of 1 mg FB_1 thrice per week, the dose was effectively 0.43 mg/kg/day. This dose compares well with the estimated 0.8 mg and 0.3 mg total fumonisins /kg body weight /day received by the two groups of monkeys in the long term exposure to *F. verticillioides* culture material at the time that their Sa/So ratios were monitored (Shephard *et al.*, 1996b). Human exposures tend to be lower than this, although it has been estimated that the rural Transkei population could be consuming 0.35 mg total fumonisins /kg body weight /day if exposed to mouldy maize as a dietary staple (Gelderblom *et al.*, 1996a). Studies at the 1 mg/kg level have been conducted in other animal species. The serum and urinary Sa/So ratios in rabbits were markedly increased over the control ratios after intravenous dosing with 1 mg FB_1 /kg weight body for five days (Gumprecht *et al.*, 1995) and by gavage for 16 days (Laborde *et al.*, 1997). In contrast to these, no significant increases were detected in serum Sa/So ratios in rats dosed with 1 mg FB_1 /kg body weight by gavage five times per week for five weeks, while the serum Sa/So ratios in mice dosed with 16.8 mg FB_1 /kg body weight by gavage three times per week for 63 weeks increased only marginally (Castegnaro *et al.*, 1998). However, urine collected from the above rats showed a 10-fold elevation in Sa/So ratio (Castegnaro et al., 1996). In contrast, no changes in urinary sphingoid base

levels were found in a study by Wang et al. (1999) on rats fed 1 mg FB_1 /kg for 60 days. Hence, the response to fumonisin exposure at low doses can vary widely between different species.

The plasma Sa/So ratios in the low- and high-FB_2-dose monkeys increased to a maximum after 7 days with the high-dose monkeys reaching higher Sa/So ratios after the single dose. The ratios remained elevated for several weeks with the low-dose monkeys returning to normal levels slightly before the high-dose animals. Compared to the previous study with FB_1, the effect of FB_2 was more sustained as the increased plasma Sa/So ratio caused by FB_1 returned to baseline levels sooner (Van der Westhuizen *et al.*, 1999). This is the first indication that FB_2, which is less polar than FB_1 due to the absence of the hydroxy group at C-10 (Fig. 1), may be more persistent in its *in vivo* effects. Persistence of the Sa/So elevation were also seen in previous studies in rat primary hepatocyte cultures once FB_1 was removed from the culture medium (Van der Westhuizen *et al.*, 1998).

In contrast to the corresponding experiment with FB_1 in which the high-dose group showed elevations in urinary Sa/So ratios, no such increases were observed in either of the FB_2 dosage groups in this study. This would suggest that FB_2 may be less nephrotoxic in monkeys than FB_1. This would correlate with toxicokinetic data which showed that FB_2 in circulation undergoes less urinary excretion in monkeys than FB_1, presumably due to its lower polarity (Shephard and Snijman, 1999). FB_1 is known to be nephrotoxic in rats (Riley *et al.*, 1994a), while toxicokinetic studies have revealed that significant amounts of the FB_1 in circulation can be eliminated in urine (Shephard *et al.*,

1992a; 1992b). Therefore it again appears that in monkeys the plasma Sa/So ratio is more effective as a biomarker of fumonisin exposure than the urinary Sa/So ratio.

As in the case with low single doses of FB_1, low single doses of FB_2 did not increase the plasma cholesterol, the liver function enzymes or the plasma renal indicators above the control levels. The effect of high doses of FB_2 on plasma cholesterol and liver function enzymes was similar to the effects seen with FB_1 in single-dose monkeys (Van der Westhuizen *et al.*, 1999). This indicates that like FB_1, FB_2 is also hepatotoxic to vervet monkeys. The lack of these biochemical effects at the low dose in comparison to the effect seen on the Sa/So ratio confirms that these nonspecific biochemical parameters are not sensitive as biomarkers of fumonisin exposure. Similar conclusions on the relative usefulness of these parameters were drawn in a study in which ponies, fed diets containing *F. proliferatum* cultures containing predominantly FB_2, caused significant disruption of sphingolipid metabolism long before any increases in the serum liver function enzymes were evident (Riley *et al.*, 1997).

This is the first report of Sa and So levels determined in plasma and urine of vervet monkeys dosed with repeated doses of pure FB_1 or a single dose of pure FB_2. This study demonstrated that similar to FB_1, a single dose of FB_2 can cause disruption of sphingoid metabolism in vervet monkeys and that repeated low doses of FB_1 can cause more severe and sustained disruption of sphingoid metabolism than either a low or high single FB_1 dose.

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Chapter 4

Sphinganine / sphingosine ratio in plasma and urine as a possible biomarker for fumonisin exposure in humans in rural areas of Africa

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ABSTRACT

This study was conducted in the Transkei region of the Eastern Cape and KwaZulu-Natal province, South Africa and in the Bomet district, western Kenya.. The sphinganine (Sa) / sphingosine (So) ratios in the plasma and urine of male and female volunteers consuming a staple diet of home-grown maize in Transkei, were 0.34 ± 0.36 (mean ± standard deviation) (n = 154) and 0.41 ± 0.72 (n = 153), respectively and in plasma samples from KwaZulu-Natal it was 0.44 ± 0.23 (n = 26). In Kenya the ratios in plasma and urine were 0.28 ± 0.07 (n = 29) and 0.34 ± 0.20 (n = 27), respectively. Mean total fumonisin level in home-grown maize, randomly collected in Transkei from the same region where the human volunteers lived, was 580 ng/g (n = 40), as compared to the KwaZulu-Natal province, where no fumonisin (n = 17) were detected (< 10 ng/g) in the home-grown maize. In Kenya, only one of seven samples was contaminated with 60 ng/g fumonisins. No significant differences were found in the Sa/So ratios between males and females within the regions nor between the different regions ($p > 0.05$). It is possible that the ratio is not sensitive enough to act as a biomarker for fumonisin exposure in humans at these levels of contamination in maize. This is the first report on Sa/So ratios determined in rural populations in Africa consuming home-grown maize as their staple diet.

Abbreviations: FB_1 = fumonisin B_1; FB_2 = fumonisin B_2; FB_3 = fumonisin B_3; HPLC = high-performance liquid chromatography; OPA = *o*-phthaldialdehyde; So = sphingosine; Sa = sphinganine.

INTRODUCTION

Ingestion of the fumonisin mycotoxins, produced by *Fusarium verticillioides* (Sacc.) Nirenberg (= *F. moniliforme* Sheldon), causes various diseases in different animal species; leukoencephalomalacia in horses (Kellerman *et al.*, 1990; Marasas *et al.*, 1988), pulmonary oedema in pigs (Harrison *et al.,* 1990) and hepatocarcinoma in rats (Gelderblom *et al.,* 1991). Fumonisins occur widely around the world in maize products intended for human consumption, raising concern over the potential hazard to human health posed by these compounds (Shephard *et al.*, 1996a; Thiel *et al.*, 1992). High levels of fumonisin contamination of home-grown maize (Rheeder *et al.,* 1992) have been correlated with the high incidence of oesophageal cancer among the maize-consuming population in the Transkei region of the Eastern Cape province, South Africa (Jaskiewicz *et al.,* 1987; Makaula *et al.,* 1996). The prevalence of oesophageal cancer in the KwaZulu-Natal province of South Africa (Van Rensburg *et al.*, 1985) and in the Bomet district of Kenya (Gatei *et al.*, 1978) is also high, but no data on fumonisin levels are available in these areas. High levels of fumonisins have also been correlated with the high incidence of oesophageal cancer in the Cixian and Linxian counties in the Republic of China (Chu and Li, 1994) and an association between fumonisin contamination and liver cancer in humans in China has been suggested (Ueno *et al.,* 1997). Based on existing data, the International Agency for Research on Cancer (IARC) has declared "toxins derived from *F. moniliforme*" to be possibly carcinogenic to humans (class 2B carcinogens) (IARC, 1993).

Toxicokinetic data have shown that fumonisin B_1 (FB_1) is eliminated rapidly from serum in vervet monkeys (Shephard *et al.*, 1994). This rapid elimination, as well as the low bioavailability and the lack of a major metabolite, implies that direct measurement of fumonisins would not be feasible as a biomarker for exposure. The primary target of the fumonisins in animal cells is sphingosine (So) [sphinganine(Sa)] *N*-acyltransferase (ceramide synthase), a key enzyme in the *de novo* sphingolipid biosynthetic pathway (Wang *et al.,* 1991). Ceramide synthase catalyses the conversion of Sa to dihydroceramide, which is then converted to ceramide via addition of the 4,5-*trans* double bond. Ceramide undergoes further metabolism to produce complex sphingolipids, such as glycosphingolipids and sphingomyelin, while So is produced as the turnover product of ceramide and other complex sphingolipids (Merrill, 1991). The inhibition of ceramide synthase leads to an elevation of Sa levels in cells, and sometimes, to a lesser extent, So levels, thus resulting in an increase in the Sa/So ratio (Riley *et al.*, 1994b). An elevation in the Sa/So ratio after the exposure to fumonisins has previously been observed in both plasma and urine of rats (Voss *et al.*, 1995), rabbits (Gumprecht *et al.*, 1995), pigs (Riley *et al.*, 1993), ponies (Wang *et al.*, 1992), mink (Morgan *et al.*, 1997) and vervet monkeys (Shephard *et al.*, 1996b). The free sphingoid bases in urine arise from the cells that accumulate in urine (Riley *et al.*, 1994a), whereas it is uncertain from which particular tissues those in serum arise as most, if not all, tissues appear to have the capacity for sphingoid base biosynthesis (Merrill *et al.*, 1993). As these changes occur before other biochemical markers of cellular injury, it has been proposed that the Sa/So ratio could be a possible biomarker for fumonisin exposure (Riley *et al.*, 1994b).

This study, aimed at determining the Sa/So ratio in plasma and urine of maize-consuming rural communities, was undertaken in the Centane (Kentani) district, Transkei region of the Eastern Cape province and Madadeni district, northern KwaZulu-Natal province of South Africa, as well as in the Bomet district, western Kenya. Sa and So levels and hence their ratios were determined in the plasma and urine of male and female volunteers, while the levels of fumonisins were determined in maize samples collected contemporaneously from these regions.

MATERIALS AND METHODS

Chemicals

Sa and So were obtained from Sigma Chemical Company (St. Louis, MO, USA). C_{20}-Sa was a generous gift from Prof. A. H. Merrill Jr, (Department of Biochemistry, Emory University, School of Medicine, Atlanta, GA, USA). All other chemicals and solvents were analytical grade from Merck (Darmstadt, Germany).

Sampling areas

Following informed consent, blood and urine samples were collected from male and female volunteers from the Centane district, Transkei, Eastern Cape and the Madadeni district, KwaZulu-Natal provinces, South Africa and the Bomet district, western Kenya. Blood only was collected from KwaZulu-Natal. In Transkei maize was sampled from 10 households in each of 4 different locations during August 1997. A random sample of

visually sound maize ('healthy' maize) was collected from each household. In addition, samples of mouldy maize were hand selected from some of these households. In KwaZulu-Natal, maize samples were collected from the households of the volunteers who donated blood (November 1995). This maize was acquired from the following sources: 17 samples were home-grown, two were bought from a farmer, one was shop bought and two were maize malt. In Kenya, seven home-grown maize samples, as well as six mouldy samples and seven maize meal samples intended for beer production, were randomly collected at households (August 1994).

Analytical methods

(i) Determination of Sa and So levels in plasma and urine

Sa and So levels in plasma and urine were determined using the method of Shephard and Van der Westhuizen (1998). The concentrations were determined by high-performance liquid chromatography (HPLC) with C_{20}- Sa as an internal standard. Plasma, obtained by centrifugation of blood at 1200 *g* for 10 min at 4°C, was deproteinised with methanol, and centrifuged. Urine or the supernatant of the deproteinised plasma was extracted by mixing with water, ammonium hydroxide and chloroform, and the 2 layers separated by centrifugation. The chloroform layer was transferred to a silica minicolumn, prepared in a polypropylene column with a lower layer of 0.5 g of silica 60 and an upper layer of anhydrous sodium sulphate (5 g). The sphingoid bases were eluted with chloroform-methanol-ammonium hydroxide (25:25:1) and dried down. The dried residue was redissolved in potassium hydroxide in methanol-chloroform (4:1) and incubated at 37°C for 1.5 hr. After hydrolysis, chloroform was

added and the solution was washed with alkaline water and the phases separated by centrifugation. The chloroform layer was dried and redissolved in 250 µl methanol, sonicated and derivatised with 50 µl OPA reagent prepared as previously described (Riley *et al.*, 1994b). A 50 µl aliquot was injected into the HPLC, which consisted of a Waters (Milford, MA, USA) Model 510 solvent delivery system, Waters U6K injector, Supelcosil ABZ+Plus (150 x 4.6 mm i.d.) column, Autochrõm APEX Integration Chromatography Workstation and Waters Fluorescence 474 detector (excitation -335 nm and emission -440 nm). The isocratic mobile phase of methanol/water (91:9) was pumped at a flow rate of 1 ml/min.

(ii) Determination of fumonisins in maize

Each collected sample was ground in a laboratory mill to a fine meal and extracted with methanol-water by homogenisation. An aliquot was applied to a strong anion exchange cartridge and the fumonisins were eluted with acetic acid in methanol. The purified extracts were evaporated to dryness, redissolved in methanol and derivatised with *o*-phthaldialdehyde (OPA). The derivatised extracts were analysed by reversed-phase HPLC using fluorescence detection (Sydenham *et al.*, 1992).

Statistical analysis

The Sa/So ratios were subjected to analysis of variance (ANOVA; one-way) between male and female groups, while the Tukey test was used to determine the statistical differences between the combined groups of the different regions.

RESULTS

The means of the concentrations of the Sa and So levels and the Sa/So ratio in the plasma of the volunteers of the three different regions are shown in Table 1 and those

Table 1. *Plasma sphinganine (Sa) and sphingosine (So) levels and the Sa/So ratio in humans from three rural areas in Africa.*

Region	Sex	No.	*Age (yr)	*Sa (nM)	*So (nM)	*Ratio Sa/So	Ratio Range
Transkei,	Male	55	48.0 ± 15.6	25.3 ± 37.9	79.1 ± 90.7	0.41 ± 0.47	0.02 - 2.97
South Africa	Female	99	48.2 ± 14.9	16.5 ± 19.3	72.2 ± 78.2	0.30 ± 0.27	0.01 - 1.46
	Combined	154	48.1 ± 15.1	19.7 ± 27.8	74.7 ± 83.0	0.34 ± 0.36	0.01 - 2.97
KwaZulu-	Male	7	59.6 ± 18.6	101 ± 121	174 ± 160	0.52 ± 0.24	0.22 - 0.88
Natal,	Female	20	44.2 ± 13.9	53.1 ± 74.5	101 ± 93.0	0.42 ± 0.22	0.12 - 0.79
South Africa	Combined	27	48.7 ± 16.6	66.8 ± 88.2	128 ± 115	0.43 ± 0.23	0.12 - 0.88
Bomet,	Male	22	43.4 ± 15.0	60.6 ± 33.4	216 ± 108	0.28 ± 0.07	0.19 - 0.50
Kenya	Female	7	45.0 ± 15.3	52.5 ± 23.3	213 ± 101	0.26 ± 0.07	0.17 - 0.38
	Combined	29	43.8 ± 14.8	58.6 ± 31.0	215 ± 105	0.28 ± 0.07	0.17 - 0.50

**Mean values ± SD. Differences between means not statistically significant ($p > 0.05$)*

in the urine in Table 2. The levels of the Sa/So ratios in the male and female volunteers were compared separately within each region for plasma and urine and there were no significant differences ($p > 0.05$). There were also no significant differences in the combined (male and female) Sa/So ratios between the different regions ($p > 0.05$). In

Table 2. *Urinary sphinganine (Sa) and sphingosine (So) levels and the Sa/So ratio in humans from two rural areas in Africa.*

Region	Sex	No.	*Age (yr)	*Sa (nM)	*So (nM)	*Ratio Sa/So	Ratio Range
Transkei,	Male	56	48.0 ± 15.6	3.08 ± 5.00	13.8 ± 21.8	0.43 ± 0.74	0.02 - 4.74
South Africa	Female	97	48.2 ± 14.9	6.32 ± 8.75	25.6 ± 32.0	0.40 ± 0.71	0.01 - 5.75
	Combined	153	48.1 ± 15.1	5.13 ± 7.75	21.3 ± 29.3	0.41 ± 0.72	0.01 - 5.75
Bomet,	Male	22	43.5 ± 14.7	1.02 ± 0.50	6.11 ± 6.97	0.34 ± 0.21	0.03 - 0.74
Kenya	Female	5	41.2 ± 15.0	3.80 ± 3.69	9.98 ± 8.54	0.34 ± 0.11	0.14 - 0.45
	Combined	27	43.0 ± 14.8	1.54 ± 1.97	6.83 ± 7.44	0.34 ± 0.19	0.03 - 0.74

**Mean values ± SD. Differences between means not statistically significant ($p > 0.050$)*

Transkei the mean level of the combined ratios measured in plasma was 0.34 ± 0.36 (standard deviation) with a range of 0.01 - 2.97 compared with KwaZulu-Natal where the mean level was 0.43 ± 0.23 with a range of 0.12 - 0.88 and in Kenya where the mean level was 0.28 ± 0.07 with a range of 0.17 - 0.50. In Transkei the mean level of the combined ratios measured in urine was 0.41 ± 0.72 with a range of 0.01 - 5.75 compared with Kenya where the mean level was 0.34 ± 0.20 with a range of 0.03 - 0.74.)

The mean level of the total fumonisins (fumonisin B_1, B_2 and B_3) in the maize collected from the households in Transkei was 580 ng/g (n = 40) in 'healthy' maize (range 0 - 7190 ng/g) and 4850 ng/g (n = 31) in 'mouldy' maize (range 30 - 37840 ng/g). The

maize collected in KwaZulu-Natal had no detectable (< 10 ng/g) levels of fumonisins. In Kenya, of the seven home-grown maize samples randomly collected, only one showed detectable contamination (> 10 ng/g) of 60 ng/g FB_1 and thus a mean of 9 ng/g in 'healthy' maize. All the mouldy samples (n = 6) showed fumonisin contamination with total fumonisin levels below 330 ng/g, with one exception which contained 5120 ng/g FB_1, 5435 ng/g FB_2 and 970 ng/g FB_3. All seven maize samples intended for beer production showed minimal contamination with FB_1 levels less than 25 ng/g. (Individual results are not shown.)

DISCUSSION

This paper reports for the first time measurements of the Sa / So ratio in human plasma and urine in three different rural populations in Africa consuming maize as their staple diet. Previous work has reported median values for the ratio in urine as 0.29 (range 0.17 - 0.53) in six healthy female volunteers in southern Italy (Solfrizzo *et al.*, 1997), whereas a second group of 14 female volunteers gave a range of values between 0.04 and 0.6 (Castegnaro *et al.*, 1996). The mean values reported here for urine collected both in Transkei and Kenya compare well with these previously reported results. Both previous studies (Castegnaro *et al.*, 1996; Solfrizzo *et al.*, 1997), although using limited numbers of subjects, failed to report values for the ratio in male volunteers due to the lower concentrations of the sphingoid bases observed in male urine. This difference in sphingoid base concentration between male and female is not observed in the present

study. Consequently, levels for male volunteers were obtained and it was observed that, as regards the ratio, no significant differences existed between male and female subjects. The only previously reported values for Sa/So ratio in the serum of human volunteers were obtained from healthy volunteers from France (eight males and 10 females) and South Africa (13 females of Asian origin), in which the values ranged from 0.09 to 0.78 (Castegnaro *et al.*, 1998), being similar to the values reported in this present study.

A number of studies have been performed on various animal species ingesting fumonisins in feed which established the Sa/So ratio as a possible biomarker for fumonisin exposure in animals. In these studies Sa and So levels were determined in plasma, urine and various tissues, in all of which an elevation in this ratio was observed due to the inhibition of the key sphingolipid biosynthetic enzyme, ceramide synthase. However, most of these studies have used relatively high levels of fumonisin exposure in order to investigate the pathological responses to fumonisin ingestion. For example, initial reports described the elevation of the ratio in the serum of ponies ingesting between 22,000 and 44,000 µg FB_1 / kg feed (Wang *et al.*, 1992). Subsequently, the disruption of sphingolipid metabolism by fumonisins was demonstrated by elevations in the ratio measured in the serum of pigs fed at levels of 1500 µg FB_1/kg body weight/day (Haschek *et al.*, 1993), in serum and urine of rabbits fed 1000 µg FB_1/kg body weight/day (Gumprecht *et al.*, 1995) and in the serum of rats fed 13,600 µg FB_1/kg body weight/day (Voss *et al.*, 1995). Recent studies in non-human primates (vervet monkeys) exposed to 'low' and 'high' levels of *F. verticillioides* culture material showed

significant elevations in the ratio in serum, with the value rising from a mean of 0.43 in controls to means of 1.72 in the 'low' dose group (ingesting 300 µg total fumonisins/kg body weight/day) and 2.57 in the high dose group (800 µg total fumonisins/kg body weight/day) (Shephard *et al.*, 1996b). Simultaneous measurements of sphingoid bases in urine of the vervet monkeys also showed elevated ratios in which the mean value in the controls of 0.87 was increased to 1.58 and 2.17 in the treated groups, respectively. However, these differences were not statistically significant due to considerable variability within each group. Unlike biomarkers such as the aflatoxin adducts, which are only present in animals or humans exposed to the toxins, the Sa/So ratio has a normal level in animals (controls) not exposed to fumonisins. Previous studies in the serum of rats, ponies and pigs consuming feed containing less than 1000 µg fumonisins/kg feed, reported ratios of 0.34, 0.20 and 0.18, respectively (Riley *et al.*, 1994b). Based on the assumption of a 200 g rat ingesting 15 g feed per day (Thiel *et al.*, 1992), these control rats were exposed to fumonisins below the level of 75 µg/kg body weight/day. In comparison to these levels of fumonisin exposure, the level of fumonisins at which carcinogenic activity was shown in rats was 3750 µg FB_1/kg body weight/day (Gelderblom *et al.*, 1991). More recent studies on a limited number of rats showed no significant change in the ratio (range 0.35 - 0.81) in serum after 5 wk of daily gavage dose of 1000 µg FB_1/kg body weight and a borderline significant elevation in serum of mice dosed by gavage with 16800 µg FB_1/kg body weight three times weekly (Castegnaro *et al.*, 1998). It is clear that considerable differences exist between species as to the sensitivity of the Sa/So ratio in either serum or urine as a biomarker of fumonisin ingestion.

In order to place the present results from plasma and urine of human volunteers in context, maize samples collected from the households in the study areas were analysed for fumonisins so as to estimate the probable daily intake (PDI) of the volunteers at the time of blood and urine collection. The mean level of total fumonisins analysed in the Transkeian maize samples was 580 ng/g, while maize collected from the volunteers in KwaZulu-Natal showed no detectable fumonisin contamination. Home-grown maize collected in Kenya showed very little fumonisin contamination, a mean total of 9 ng/g, which was consistent with a previous report on low levels of *F. verticillioides* infestation of maize in western Kenya (MacDonald and Chapman, 1997). Based on a mean fumonisin contamination of 580 ng/g in 'healthy' maize in Transkei and the assumption that a 70 kg person reliant on maize as the staple diet, consumes 460 g maize per day (Langenhoven *et al.*, 1988), then the PDI of fumonisins by the volunteers in Transkei who donated blood and urine, was 3.8 µg/kg body weight/day. The volunteers in KwaZulu-Natal were not exposed to fumonisins and in Kenya, based on a mean fumonisin contamination of 9 ng/g in 'healthy' maize, the volunteers there would be exposed to a mean PDI of around 0.06 µg/kg body weight/day. This figure is comparable to the PDI of some populations in western Europe (Shephard *et al.*, 1996a). This is extremely low and, similarly to the study population in KwaZulu-Natal, these Kenyan volunteers may be regarded as a control group.

Previous studies in Transkei have shown a high incidence of *F. verticillioides* infestation in home-grown maize, accompanied by high levels of fumonisin contamination (Rheeder *et al.*, 1992). During the 1985 and 1989 seasons, 'healthy' maize from

Transkei was shown to have mean levels of total fumonisins in all samples analysed of 2100 ng/g and 1630 ng/g, respectively. The PDI values for 1985 and 1989, based on the previous assumptions, were 13.8 µg/kg body weight/day and 10.7 µg/kg body weight/day, respectively compared to 3.8 µg/kg body weight/day for 1997. These values are considerably higher than the recently proposed tolerable daily intake of 0.8 µg/kg body weight/day (Gelderblom *et al.*, 1996; Marasas 1997). The mean Sa/So ratio in plasma and urine in these volunteers was not significantly different from the groups designated above as controls, and it is possible that the ratio is not sensitive enough to act as a biomarker for fumonisin exposure at these PDI levels. However, the maximum levels of the of the Sa/So ratio in plasma in males and females in Transkei (2.97 and 1.46, respectively) are higher than those of KwaZulu-Natal (0.88 and 0.79, respectively) and Kenya (0.50 and 0.38). It is also true for the urine values in males and females in Transkei (4.74 and 5.75, respectively) compared to Kenya (0.74 and 0.45). In contrast to the doses at which elevations in the ratio have been observed in animals, the PDI values for the population of Transkei are around 2 orders of magnitude lower.

The analysis of the data from 154 volunteers from Transkei suggests that the Sa/So ratio may be only of limited value as a biomarker of fumonisin exposure. It certainly is applicable in animal studies in which levels of exposure are relatively high, but the PDI of fumonisins in most human populations is considerably lower. One possible exception would be found, should the 'mouldy' maize of the Transkei region be consumed as the staple diet, at which time the PDI levels would increase considerably (Gelderblom *et al.*, 1996). A further complication in the potential use of the ratio is in determining the

normal levels of a control group, as all the studies conducted to date in humans and animals alike, have demonstrated a considerable variation in the ratio between individuals and within an individual over time.

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Summary

In investigating the structural requirements for ceramide synthase inhibition in rat primary hepatocyte cultures, it was found that the AAL-toxins, TA and TB, which have only one tricarballylic acid (TCA) group, increased the Sa/So ratios to a similar degree as FB_1, while AP_1, the complete hydrolysis product of FB_1, increased the Sa/So ratio to a much lesser extent than FB_1. It therefore appears that at least one TCA group is required for maximal ceramide synthase inhibition. As FA_1, the *N*-acetyl derivative of FB_1, also increased the Sa/So ratio to a similar degree as FB_1, it seems that the presence of a free amino-group is not a requisite for enzyme inhibition. The structural similarity in the head groups of the sphingoid bases and the toxins allow them to be competitive inhibitors of the enzyme and the tricarballylic acid moieties might also interact with the binding site for the fatty acid moiety. Furthermore it was found that the inhibition of ceramide synthase in primary rat hepatocytes was irreversible due the persistence of the increase in the Sa/So ratios after removal of FB_1 from the incubation media.

The extent to which sphingolipid biosynthesis was affected was correlated with the respective cytotoxicities of the structural analogues *in vitro*. Regarding FB_1 and AP_1, the absence of the TCA moieties increased the cytotoxic effect in primary hepatocytes, possibly due to a decrease in the polarity of the molecule. In the case of TA and TB the presence of a single TCA moiety seems not to correlate with cytotoxicity as TA is more cytotoxic than TB while both toxins are less cytotoxic than FB_1. In this regard other

structural differences between the fumonisins and TA and TB could also play a contributing role. The contrasting results between cytotoxicity and the elevation of Sa/So ratios in primary hepatocytes, indicate that the cytotoxicity of these compounds is not solely due to inhibition of ceramide synthase and the subsequent changes in sphingoid base concentrations. It would thus appear that the structural requirements for cytotoxicity and inhibition of ceramide synthase differ in primary hepatocytes.

The seven day FB_1 gavage study in vervet monkeys indicated that the disruption of sphingolipid metabolism, as evidenced by elevated serum Sa/So ratios, showed no sign of abatement after the single low and high gavage doses. In addition, key chemical pathological parameters of liver damage and renal function also showed no abatement during this period. The determination of Sa/So ratio in the liver and kidney tissues indicated that a dose-dependent effect on sphingolipid metabolism occurred in both organs and that hence both liver and kidney appear to be target organs for FB_1 in vervet monkeys.

The serum Sa/So ratios in the 50-day FB_1 gavage study increased significantly within the first week of the study period in low- and high-dose monkeys and remained elevated over an extended period. Urinary Sa/So ratios showed a more rapid response and a correspondingly more rapid return to pre-dosing levels. The chemical pathology parameters monitored for liver damage and renal function were only affected at the high dose and the leakage of ALT, AST, LDH and GGT into the serum is indicative of hepatotoxicity. Serum creatinine and urea, the renal function indicators, had the same

rapid transient response as the urinary Sa/So ratio in the high dose-monkeys, indicating nephrotoxicity. It would thus seem that the serum Sa/So ratio is more sensitive to the low dose effects of FB_1 than the other serum indicators, since the other serum indicators showed a sustained elevation only at the high dose. Despite the low bioavailability and rapid elimination of FB_1 in serum, the biochemical changes that it induces are of an extended nature such that a single large exposure can still have measurable effects many weeks later.

Repeated FB_1 doses in vervet monkeys at the low dose level of the single dose experiment resulted in the sustained elevation of plasma Sa/So ratios and blood biochemistry parameters (liver enzymes and cholesterol). The lack of a clear elevation in urinary Sa/So ratio after 51 days of multiple exposure indicates that in monkeys, the plasma ratio is more sensitive than urinary changes. In this regard, no indications were found in plasma of renal toxicity from the repeated doses, as urea and creatinine levels failed to rise to abnormal levels. This study demonstrated that repeated low doses of FB_1 can cause more severe and sustained disruption of sphingoid metabolism than a single FB_1 dose at either a low or high level.

The plasma Sa/So ratios in the low- and high-FB_2-dose monkeys increased with the high-dose monkeys reaching higher Sa/So ratios after the single dose. The ratios remained elevated for several weeks with the low-dose monkeys returning to normal levels slightly before the high-dose animals. Compared to the previous study with FB_1, the effect of FB_2 was more sustained as the increased plasma Sa/So ratio caused by

FB_1 returned to baseline levels sooner. This is the first indication that FB_2, which is less polar than FB_1 due to the absence of the hydroxy group at C-10, may be more persistent in its *in vivo* effects. In contrast to the corresponding experiment with FB_1 in which the high-dose group showed elevations in urinary Sa/So ratios, no such increases were observed in either of the FB_2 dosage groups in this study. This would suggest that FB_2 may be less nephrotoxic in monkeys than FB_1 and therefore it again appears that in monkeys the plasma Sa/So ratio is more effective as a biomarker of fumonisin exposure than the urinary Sa/So ratio. As in the case with low single doses of FB_1, low single doses of FB_2 did not increase the plasma cholesterol, the liver function enzymes or the plasma renal indicators (creatinine and urea) above the control levels. The effect of high doses of FB_2 on plasma cholesterol and liver function enzymes is similar to the effects seen with FB_1 in single-dose monkeys. This indicates that like FB_1, FB_2 is also hepatotoxic to vervet monkeys. The lack of these biochemical effects at the low dose in comparison to the effect seen on the Sa/So ratio confirms that these nonspecific biochemical parameters are not sensitive as biomarkers of fumonisin exposure. This study demonstrated that similar to FB_1, a single dose of FB_2 can cause disruption of sphingoid metabolism in vervet monkeys.

This is the first report of measurements of the Sa/So ratio in human plasma and urine in different rural populations in Africa consuming maize as their staple diet and where the mean level of total fumonisins were analysed in maize samples collected from the households in the same study areas. No significant differences were found in the mean plasma and urinary Sa/So ratios between male and female subjects nor between the

combined ratios of the different regions. However, the upper levels of the ranges of the Sa/So ratios in volunteers in the Transkei, where significantly higher levels of fumonisins were found in the maize, were much higher than the upper ranges of the ratios in the other two areas. The analysis of the data from the human volunteers from these areas in Africa suggests that the Sa/So ratio may be only of limited value as a biomarker of fumonisin exposure. Further complications in the potential use of the ratio are in determining the normal levels of a control group and the considerable variation in the ratio between individuals and within an individual over time.