AFLATOXINS

These substances were considered by previous working groups, in December 1971 (IARC, 1972), October 1975 (IARC, 1976), March 1987 (IARC, 1987) and June 1992 (IARC, 1993). Since that time, new data have become available, and these have been incorporated into this updated monograph.

1. Exposure Data

1.1 Chemical and physical data

1.1.1 *Synonyms, structural and molecular data* (see Figure 1)

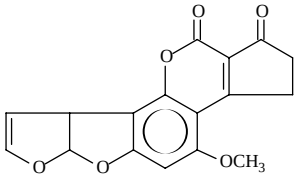
Aflatoxin B_1

Chem. Abstr. Services Reg. No.: 1162-65-8
Deleted CAS Nos: 13214-11-4; 11003-08-0; 27261-02-5
Chem. Abstr. Name: (6aR,9aS)-2,3,6a,9a-Tetrahydro-4-methoxycyclopenta[*c*]furo-(3′,2′:4,5)furo[2,3-*h*][*l*]benzopyran-1,11-dione (9CI)
Synonyms: 6-Methoxydifurocoumarone; 2,3,6aα,9aα-tetrahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione; (6aR-*cis*)-2,3,6a,9a-tetrahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione

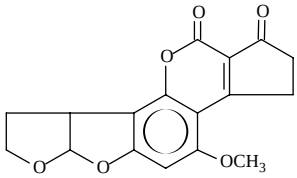
Aflatoxin B_2

Chem. Abstr. Services Reg. No.: 7220-81-7
Chem. Abstr. Name: (6aR,9aS)-2,3,6a,8,9,9a-Hexahydro-4-methoxycyclopenta[*c*]-furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione (9CI)
Synonyms: Dihydroaflatoxin B_1; 2,3,6aα,8,9,9aα-hexahydro-4-methoxycyclopenta-[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione; (6aR-*cis*)-2,3,6a,8,9,9a-hexahydro-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione

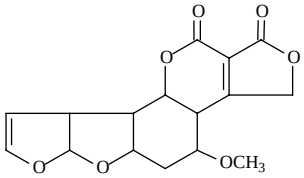
Figure 1. Structures of naturally occurring aflatoxins



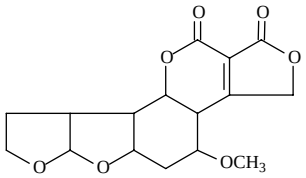
B_1: $C_{17}H_{12}O$ Mol. wt: 312.3



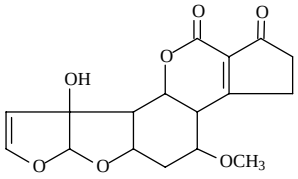
B_2: $C_{17}H_{14}O_6$ Mol. wt: 314.3



G_1: $C_{17}H_{12}O_7$ Mol. wt: 328.3



G_2: $C_{17}H_{14}O_7$ Mol. wt: 330.3



M_1: $C_{17}H_{12}O_7$ Mol. wt: 328.3

Aflatoxin G_1

Chem. Abstr. Services Reg. No.: 1165-39-5
Deleted CAS No.: 1385-95-1
Chem. Abstr. Name: (7aR,10aS)-3,4,7a,10a-Tetrahydro-5-methoxy-1*H*,12*H*-furo-[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione (9CI)
Synonym: 3,4,7aα,10aα-Tetrahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]-pyrano-[3,4-*c*][*l*]benzopyran-1,12-dione; (7aR-*cis*)-3,4,7a,10a-tetrahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione

Aflatoxin G_2

Chem. Abstr. Services Reg. No.: 7241-98-7
Chem. Abstr. Name: (7aR,10aS)-3,4,7a,9,10,10a-Hexahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione (9CI)
Synonyms: Dihydroaflatoxin G_1; 3,4,7aα,9,10,10aα-hexahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]furo[2,3-*h*]pyrano[3,4-*c*][*l*]benzopyran-1,12-dione; (7aR-*cis*)-3,4,7a,9,10,10a-hexahydro-5-methoxy-1*H*,12*H*-furo[3′,2′:4,5]-furo[2,3-*h*]pyrano[3,4-*c*][*l*]-benzopyran-1,12-dione

Aflatoxin M_1

Chem. Abstr. Services Reg. No.: 6795-23-9
Chem. Abstr. Name: (6aR,9aR)-2,3,6a,9a-Tetrahydro-9a-hydroxy-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione (9CI)
Synonym: 4-Hydroxyaflatoxin B_1; (6aR-*cis*)-2,3,6a,9a-tetrahydro-9a-hydroxy-4-methoxycyclopenta[*c*]furo[3′,2′:4,5]furo[2,3-*h*][*l*]benzopyran-1,11-dione

1.1.2 *Chemical and physical properties of aflatoxins* (from Castegnaro *et al.*, 1980, 1991; O'Neil *et al.*, 2001, unless otherwise stated)

(*a*) *Description*: Colourless to pale-yellow crystals. Intensely fluorescent in ultraviolet light, emitting blue (aflatoxins B_1 and B_2) or green (aflatoxin G_1) and green–blue (aflatoxin G_2) fluorescence, from which the designations B and G were derived, or blue–violet fluorescence (aflatoxin M_1)
(*b*) *Melting-points*: see Table 1.
(*c*) *Absorption spectroscopy*: see Table 1.
(*d*) *Solubility*: Very slightly soluble in water (10–30 µg/mL); insoluble in non-polar solvents; freely soluble in moderately polar organic solvents (e.g., chloroform and methanol) and especially in dimethyl sulfoxide (Cole & Cox, 1981)
(*e*) *Stability*: Unstable to ultraviolet light in the presence of oxygen, to extremes of pH (< 3, > 10) and to oxidizing agents
(*f*) *Reactivity*: The lactone ring is susceptible to alkaline hydrolysis. Aflatoxins are also degraded by reaction with ammonia or sodium hypochlorite.

1.1.3 *Analysis*

Methods for determining aflatoxins in agricultural commodities and food products have been verified by AOAC International (IARC, 1993; AOAC International, 2000; Stroka *et al.*, 2001) and by various international committees (ISO, 1998; EN, 1999a,b; ISO, 2001), as shown in Table 2. The methods have greatly improved in recent years with the commercial availability of multifunctional columns and immunoaffinity

Table 1. Melting-points and ultraviolet absorption of aflatoxins

Aflatoxin	Melting-point (°C)	Ultraviolet absorption (ethanol)	
		λ_{max} (nm)	ε (L mol^{-1} cm^{-1})
B_1	268–269 (decomposition)	223	25 600
	(crystals from chloroform)	265	13 400
		362	21 800
B_2	286–289 (decomposition)	265	11 700
	(crystals from chloroform-pentane)	363	23 400
G_1	244–246 (decomposition)	243	11 500
	(crystals from chloroform-methane)	257	9 900
		264	10 000
		362	16 100
G_2	237–240 (decomposition)	265	9 700
	(crystals from ethyl acetate)	363	21 000
M_1	299 (decomposition)	226	23 100
	(crystals from methanol)	265	11 600
		357	19 000

From O'Neil *et al.* (2001)

columns, which are simple and rapid to use, and with reduction in the use of toxic solvents for extraction and clean-up.

Quality assurance for the analysis of aflatoxins B_1, B_2, G_1, G_2 and M_1 in foods is available for laboratories through the American Association of Cereal Chemists' Check Sample Program and the Analytical Proficiency Testing Programme administered in the USA and the United Kingdom, respectively.

As contamination may not occur in a homogeneous way throughout a sample of maize or peanuts[1], good sampling and sample preparation procedures must be used to obtain accurate quantitative results. Summaries of the procedures, variability and application of sampling plans for mycotoxins are included in Section 1.5 and in the European Commission directive 98/53/CE (European Commission, 1998a).

A number of approaches have been used to analyse aflatoxins and their metabolites in human tissues and body fluids. These include immunoaffinity purification, immunoassay (Wild *et al.*, 1987), high-performance liquid chromatography (HPLC) with fluorescence or ultraviolet detection and synchronous fluorescence spectroscopy (Groopman & Sabbioni, 1991). Molecular biomarkers, such as urinary markers, metabolites in milk and parent compounds in blood, are used for determining exposure to aflatoxins (Groopman, 1993).

[1] Maize (corn) and peanuts (groundnuts) will be used throughout this volume for corn and groundnuts.

Table 2. Analytical methods validated by AOAC International and the EU

Method no.	Aflatoxin	Food	Method[a]	Detection limit (μg/kg)
AOAC				
975.36	All[b]	Food and feeds (screening)	MC	5–15
979.18	All	Maize and peanuts (screening)	MC	10
990.31	All	Maize and peanuts (Aflatest screening)	IC	10
994.08	B_1, B_2, G_1, G_2	Maize, almond, Brazil nuts, peanuts, pistachio nuts (Mycosep)	MFC/HPLC	5
999.07	All, B_1	Peanut butter, pistachio paste, fig paste, paprika powder	IC/HPLC	NG
989.06	B_1	Cottonseed products and mixed feed (screening)	ELISA	15
990.32	B_1	Maize and roasted peanuts (screening)	ELISA	20
2000.16	B_1	Baby foods (infant formula)	IC/HPLC	0.1
990.34	B_1, B_2, G_1	Maize, cottonseed, peanuts, peanut butter (screening)	ELISA	20–30
991.45	B_1, B_2, G_1, G_2	Peanut butter	ELISA	9
993.16	B_1, B_2, G_1	Maize	ELISA	20
998.03	B_1, B_2, G_1, G_2	Peanuts	TLC	NG
968.22	B_1, B_2, G_1, G_2	Peanuts and peanut products	TLC	5
970.45	B_1, B_2, G_1, G_2	Peanuts and peanut products	TLC	10
971.23	B_1, B_2, G_1, G_2	Cocoa beans	TLC	10
971.24	B_1, B_2, G_1, G_2	Coconut, copra and copra meal	TLC	50
972.26	B_1, B_2, G_1, G_2	Maize	TLC	5
980.20	B_1, B_2, G_1, G_2	Cottonseed products and mixed fed (screening)	TLC, HPLC	10, 5
974.16	B_1, B_2, G_1, G_2	Pistachio nuts	TLC	15
972.27	B_1, B_2, G_1, G_2	Soya bean	TLC	10
990.33	B_1, B_2, G_1, G_2	Maize and peanut butter	HPLC	5
993.17	B_1, B_2, G_1, G_2	Maize and peanuts	TLC	1.5–10
991.31	B_1, B_2, G_1, G_2	Maize, peanuts, peanut butter (Aflatest)	IC/HPLC	10
970.46	B_1, B_2, G_1, G_2	Green coffee	TLC	25
978.15	B_1	Eggs	TLC	0.1
982.24	B_1 and M_1	Liver	TLC	0.1
974.17	M_1	Dairy products	TLC	0.1
980.21	M_1	Milk and cheese	TLC	0.1
986.16	M_1 and M_2	Fluid milk	HPLC	0.1

Table 2 (contd)

Method no.	Aflatoxin	Food	Method[a]	Detection limit (µg/kg)
EU				
NF EN 12955	All, B_1	Cereals, nuts and derived products	IC/HPLC	8 (all)
NF EN ISO 14501	M_1	Milk and milk powder	IC/HPLC	0.08 in powder 0.008 µg/L liquid
ISO 14718	B_1	Mixed feeding stuff	HPLC	1
ISO 6651	B_1	Animal feeding stuff	TLC/fluo-rescence	4

From IARC (1993); ISO (1998); EN (1999a,b); AOAC International (2000); ISO (2001); Stroka *et al*. (2001)

[a] MC, minicolumn; IC, immunoaffinity column, ELISA, enzyme-linked immunosorbent assay; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; MFC, multifunctional column

[b] All, sum or total aflatoxins

AOAC, Association of Analytical Communities; EU, European Union; NG, not given

1.2 Sources, production and use

1.2.1 *Fungi producing aflatoxins*

Aflatoxins are produced by the common fungi *Aspergillus flavus* and the closely related species *A. parasiticus*. These are well defined species: *A. flavus* produces only B aflatoxins and sometimes the mycotoxin cyclopiazonic acid (CPA), while *A. parasiticus* produces both B and G aflatoxins, but never CPA (Schroeder & Boller, 1973; Dorner *et al.*, 1984; Klich & Pitt, 1988; Pitt, 1993) (see Annex).

This simple situation, of just two aflatoxigenic species, has been complicated by more recent taxonomic findings. Kurtzman *et al.* (1987) described *A. nomius*, a species closely related to *A. flavus* but which produces small bullet-shaped sclerotia, as distinct from the large spherical sclerotia produced by many *A. flavus* isolates. This species is also distinguished from *A. flavus* by the production of both B and G aflatoxins (Saito *et al.*, 1989; Pitt, 1993). A second new species, closely related to *A. nomius*, was described by Peterson *et al.* (2001) and named *A. bombycis*. These two species were distinguished from each other by differences in DNA, and also by differences in growth rates at 37 °C. Like *A. nomius*, *A. bombycis* produces both B and G aflatoxins.

The species *A. ochraceoroseus* described by Bartoli and Maggi (1978) was recently shown to be another aflatoxin producer. It also produces sterigmatocystin (Frisvad, 1997; Klich *et al.*, 2000). Saito and Tsuruta (1993) described *A. flavus* var. *parvisclerotigenus*, which produces small spherical sclerotia, but one isolate (NRRL 3251) (Agricultural Research Service Culture Collection) reported to be representative of the new variety was considered by both Christensen (1981) and Pitt (1993) to be a typical *A. flavus*. This same isolate was reported by Stubblefield *et al.* (1970) to produce B but not G aflatoxins, in line with those assessments. Moreover, Geiser *et al.* (2000) showed that the production of small versus large sclerotia does not have taxonomic significance within *A. flavus*.

Two aflatoxin-producing isolates from Japan, originally classified as aberrant *A. tamarii* (Goto *et al.*, 1996), were recently described as *A. pseudotamarii*. Like *A. flavus*, this species produces B aflatoxins and CPA, but differs from *A. flavus* by the production of orange-brown conidia (Ito *et al.*, 2001).

In studying population genetics of *A. flavus*, Geiser *et al.* (1998) showed that *A. flavus* from an Australian peanut field comprised two distinct subgroups, which they termed Group I and Group II, and suggested that Group II differed from Group I (*A. flavus sensu stricto*) sufficiently to be raised to species level. Further studies by Geiser *et al.* (2000) and independent observations have confirmed that *A. flavus* Group II comprises a distinct species, which will be described as '*Aspergillus australis*'. Unlike any other known species, *A. australis* produces both B and G aflatoxins and also CPA. It appears to occur almost exclusively in the southern hemisphere, where it has been found in Argentina, Australia, Indonesia and South Africa.

The current status of taxonomic information and mycotoxin production by species that produce aflatoxins is summarized in Table 3. This information, complex though it is, should not be allowed to obscure the importance of the older species. The evidence

Table 3. *Aspergillus* species capable of producing aflatoxins

Species	Mycotoxins produced			Major sources	Geographical distribution
	AFB	AFG	CPA		
A. flavus	+	–	–	All kinds of foods	Ubiquitous in warmer latitudes
A. parasiticus	+	+	–	Peanuts	Specific areas
A. nomius	+	+	–	Bees	USA, Thailand
A. pseudotamarii	+	–	+	Soil	Japan
A. bombycis	+	+	–	Silkworm frass	Japan, Indonesia
A. ochraceoroseus	+	–	–	Soil	Africa
A. australis	+	+	+	Soil, peanuts	Southern hemisphere

AFB, B aflatoxins; AFG, G aflatoxins; CPA, cyclopiazonic acid

indicates that *A. flavus* and *A. parasiticus* are responsible for the overwhelming proportion of aflatoxins found in foodstuffs throughout the world. Of the other species, only *A. australis*, which appears to be widespread in the southern hemisphere and is common in Australian peanut soils, may also be an important source of aflatoxins in a few countries.

1.2.2 *Production and reduction*

Apart from natural formation, aflatoxins are produced only in small quantities for research purposes, by *A. flavus* or *A. parasiticus* fermentations on solid substrates or media in the laboratory. Aflatoxins are extracted by solvents and purified by chromatography. Total annual production is less than 100 g (IARC, 1993).

Aflatoxins occurring naturally in foods and feeds may be reduced by a variety of procedures. Improved farm management practices, more rapid drying and controlled storage are now defined within GAP (Good Agricultural Practice) or HACCP (Hazard Analysis: Critical Control Point) (FAO, 1995). By segregation of contaminated lots after aflatoxin analyses and by sorting out contaminated nuts or grains by electronic sorters, contaminated lots of peanuts or maize can be cleaned up to produce food-grade products. Decontamination by ammoniation or other chemical procedures can be used for rendering highly contaminated commodities suitable as animal feeds. More detailed information on these topics is given in the Annex to this Monograph.

1.2.3 *Uses*

Aflatoxins are not used commercially, only for research.

1.3 Formation and occurrence

1.3.1 *Prevalence of toxigenic species in foods*

Because of the importance of aflatoxins, *A. flavus* has become the most widely reported foodborne fungus — even with the proviso that *A. parasiticus* is sometimes not differentiated from *A. flavus* in general mycological studies. *A. flavus* is especially abundant in the tropics. Levels of *A. flavus* in warm temperate climates such as in the USA and Australia are generally much lower, while the occurrence of *A. flavus* is uncommon in cool temperate climates except in foods and feeds imported from tropical countries (see Section 1.3.3).

The major hosts of *A. flavus* among food and feed commodities are peanuts, maize and cottonseed. In addition, various spices sometimes contain aflatoxins, while tree nuts are contaminated less frequently. Low levels may be found in a wide range of other foods (Pitt *et al*., 1993, 1994; Pitt & Hocking, 1997)

It seems probable that although *A. parasiticus* has the same geographical range as *A. flavus*, it is less widely distributed. In particular, it has been found only rarely in south-east Asia. The food-related hosts of *A. parasiticus* are similar to those of *A. flavus*, except that *A. parasiticus* is very uncommon in maize (Pitt *et al*., 1993, 1994).

1.3.2 *Factors affecting formation of aflatoxins in foods*

A fundamental distinction must be made between aflatoxin formation in crops before (or immediately after) harvest, and that occurring in stored commodities or foods. Peanuts, maize and cottonseed are associated with *A. flavus*, and in the case of peanuts, also with *A. parasiticus*, so that invasion of plants and developing seed or nut may occur before harvest. This close association results in the potential for high levels of aflatoxins in these commodities and is the reason for the continuing difficulty in eliminating aflatoxins from these products.

In contrast, *A. flavus* lacks this affinity for other crops, so it is not normally present at harvest. Prevention of the formation of aflatoxins therefore relies mainly on avoidance of contamination after harvest, using rapid drying and good storage practice (see Annex).

1.3.3 *Occurrence*

Aflatoxins have been found in a variety of agricultural commodities, but the most pronounced contamination has been encountered in maize, peanuts, cottonseed and tree nuts. Aflatoxins were first identified in 1961 in animal feed responsible for the deaths of 100 000 turkeys in the United Kingdom (Sargeant *et al*., 1961). An extensive review of the levels of aflatoxins encountered in commodities in North America, South America, Europe, Asia and Africa was included in the previous IARC monograph (IARC, 1993).

A summary of data published since the previous monograph on the worldwide occurrence of aflatoxins is given in Table 4. From the point of view of dietary intake,

Table 4. Occurrence of aflatoxin B_1 in Latin America and Asia

Product	Region/Country	Detected/total no. of samples	Aflatoxin B_1 (μg/kg)	Compiled by the Working Group from the following references
	Latin America			
Maize	Argentina, Brazil, Costa Rica, Mexico, Venezuela	5086/15 555	0.2–560	Viquez *et al*. (1994); Torres Espinosa *et al*. (1995); Juan-López *et al*. (1995); Resnik *et al*. (1996); González *et al*. (1999) (none found in Argentina); Medina-Martínez & Martínez (2000); Ono *et al*. (2001); Vargas *et al*. (2001)
Maize foods	Brazil	30/322	2.80–1323[a]	Midio *et al*. (2001)
Peanuts and products	Brazil	41/80	Max. 1789	Freitas & Brigido (1998)
Soya bean	Argentina	9/94	< 1–11	Pinto *et al*. (1991)
Sorghum	Brazil	18/140	7–33 (mean)	da Silva *et al*. (2000)
Poultry feed	Argentina	41/300	17–197	Dalcero *et al*. (1997)
	Asia			
Maize and flour	China, India, Indonesia, Philippines, Thailand	1263/2541	0.11–4030	Yamashita *et al*. (1995); Yoshizawa *et al*. (1996); Zhang *et al*. (1996); Bhat *et al*. (1997); Ueno *et al*. (1997); Shetty & Bhat (1997); Ali *et al*. (1998); Vasanthi & Bhat (1998); Lipigorngoson *et al*. (1999); Li *et al*. (2001)
Maize products	Malaysia, Philippines	77/404	1–117	Ali *et al*. (1999); Arim (2000)
Maize feed	Viet Nam	27/32	8.6–96.0	Wang *et al*. (1995)
Peanuts	China, India, Japan, Thailand	1456/7796	0.2–833	Bhat *et al*. (1996); Zhang *et al*. (1996); Hirano *et al*. (1998); Lipigorngoson *et al*. (1999); Okano *et al*. (2002)
Peanut products, oil, butter	China, Malaysia, Philippines	235/594	1–244	Zhang *et al*. (1996); Ali *et al*. (1999); Arim (2000)

Table 4 (contd)

Product	Region/Country	Detected/total no. of samples	Aflatoxin B_1 (μg/kg)	Compiled by the Working Group from the following references
Peanut foods	India, Malaysia, Philippines	177/957	1–1500	Rati & Shantha (1994); Ali (2000); Arim (2000)
Nuts and products	Japan	23/673	0.3–128	Tabata *et al*. (1998)
Rice and wheat	China	0/92		Zhang *et al.* (1996)
Sorghum	India, Thailand	56/94	0.10–30.3	Shetty & Bhat (1997); Suprasert & Chulamorakot (1999)
Commercial foods	Japan, Malaysia	154/1053	0.1– > 50[a]	Taguchi *et al*. (1995); Tabata *et al*. (1998); Ali (2000)
Beer	Japan	13/116	0.0005–0.0831	Nakajima *et al*. (1999)

[a] Total aflatoxins

aflatoxins in foods used as staples such as maize assume considerable significance. Aflatoxins are a far greater problem in the tropics than in temperate zones of the world. However, because of the movement of agricultural commodities around the globe, no region of the world is free of aflatoxins.

With regard to aflatoxin contamination in foods imported into Japan, relatively low incidences and low levels of aflatoxins have been found in various commodities. Aflatoxin inspection of imported peanuts (1999–2000) indicated that 355 (6.9%) of 5108 samples were contaminated with aflatoxin B_1 at levels ranging from 0.2 to 760 μg/kg, and 145 samples (2.8%) contained over 10 μg/kg, the maximum permitted level in Japan (Okano *et al.*, 2002). In commercial nuts and nut products in markets, aflatoxin B_1 was found in 23 (3.4%) of 673 samples at levels of 0.3–128 μg/kg. Imported spices (white and red pepper, paprika and nutmeg) contained aflatoxin B_1 in 106 (19.4%) of 546 samples at levels of 0.2–27.7 μg/kg (Tabata *et al.*, 1998).

Information on the occurrence of aflatoxins in imported spices in the European Union (EU) is given in Table 5. Among the total of 3098 spice samples including nutmeg, pepper, chilli and paprika, 183 samples (5.9%) contained more than 10 μg/kg aflatoxins (European Commission, 1997).

Table 5. Aflatoxin B_1 in spices imported into the European Union

Product	Detected/ total samples	Aflatoxin B_1 (μg/kg)	
		> 2	> 10
Nutmeg	333/546	25%	8%
Pepper	282/828	7%	1%
Chilli and chilli powder	148/509	28%	9%
Paprika powder	195/1215	21%	7%
Total spices	958/3098	> 1 μg/kg	
	591/3098	> 2 μg/kg	
	183/3098	> 10 μg/kg	

From European Commission (1997)

In the United Kingdom, seven of 139 maize samples (5.0%) imported in 1998–99 contained total aflatoxins in the range of 4.9–29.1 μg/kg (3.7–16.4 μg/kg aflatoxin B_1) (MAFF, 1999).

The French Direction Générale de la Concurrence, de la Consommation et de la Répression des Fraudes (DGCCRF) surveyed 635 imported foods between 1992 and 1996, of which 227 (35.7%) had aflatoxin B_1 levels above 0.05 μg/kg. The highest levels were found in spices (up to 75 μg/kg) and dried fruits (up to 77 μg/kg) (Castegnaro & Pfohl-Leszkowicz, 1999).

Dietary intake of aflatoxin B_1 was monitored for one week in a number of households in a Chinese village. Aflatoxin B_1 was detected in 76.7% (23/30) of ground maize samples (range, 0.4–128.1 μg/kg), 66.7% (20/30) of cooking peanut oil samples (range, 0.1–52.5 μg/L) and 23.3% (7/30) of rice samples (range, 0.3–20 μg/kg) (Wang *et al.*, 2001).

(*a*) *Co-occurrence of aflatoxins and fumonisins*

Co-occurrence of aflatoxin B_1 and fumonisin B_1 in maize and sorghum from Latin America and Asia is shown in Table 6. Maize harvested in the tropical and subtropical areas of the world with hot and humid climates is the major commodity contaminated with the two mycotoxins.

Two studies were carried out on cross-contamination with aflatoxins and fumonisins in staple maize samples from two high-risk areas for human hepatocellular carcinoma in China; Haimen, Jiangsu Province, Shandong (Ueno *et al.*, 1997) and Chongzuo County, Guangxi in 1998 (Li *et al.*, 2001). Three-year (1993–95) surveys demonstrated that maize harvested in Haimen was highly contaminated with aflatoxins and fumonisins and that the levels of fumonisins were 10–50-fold higher than in a low-risk area (Ueno *et al.*, 1997). Staple maize samples from Guangxi were co-contaminated (14/20) with high levels of aflatoxin B_1 (11–2496 μg/kg) and fumonision B_1 (74–780 μg/kg), and the probable daily intake was estimated to be 3.68 μg/kg bw of aflatoxin B_1 and 3.02 μg/kg bw of fumonisin B_1 (Li *et al.*, 2001).

In India, rain-affected maize samples from rural households and retail shops had higher levels of contamination with fumonisins (250–6470 μg/kg) than normal samples (50–240 μg/kg) as well as with aflatoxin B_1 (250–25 600 versus 5–87 μg/kg), which co-occurred with fumonisins. The level of fumonisin B_1 was also higher in sorghum affected by rain (140–7800 μg/kg versus 70–360 μg/kg). No correlation was observed between levels of the two toxins in individual samples, indicating that the toxins are formed independently (Vasanthi & Bhat, 1998).

(*b*) *Occurrence of aflatoxin M_1*

Aflatoxin M_1 is a metabolite of aflatoxin B_1 that can occur in milk and milk products from animals consuming feed contaminated with B aflatoxins (Applebaum *et al.*, 1982). Data on occurrence of aflatoxin M_1 in milk were summarized earlier (IARC, 1993) and data reported subsequently are included in Table 7.

Galvano *et al.* (1996) reviewed the worldwide occurrence of aflatoxin M_1 in milk and milk products.

1.3.4 *Human biological fluids*

Covalent binding of aflatoxin to albumin in peripheral blood has been measured in a number of studies (Montesano *et al.*, 1997). The levels of these adducts are assumed to reflect exposure to aflatoxin over the previous 2–3 months, based on the half-life of

Table 6. Co-occurrence of aflatoxins and fumonisins in Asia and Latin America

Product	Region/Country	No. detected/total no. of samples			Range (μg/kg)		Compiled by the Working Group from the following references
		AFB_1	FB_1	AFB_1 + FB_1	AFB_1	FB_1	
	Asia						
Maize	China, India, Indonesia, Philippines, Thailand, Viet Nam	199/234	173/234	148/234	0.11–4030	10–18 800	Yamashita *et al*. (1995); Wang *et al.* (1995); Shetty & Bhat (1997); Ueno *et al*. (1997); Ali *et al*. (1998); Vasanthi & Bhat (1998); Li *et al*. (2001)
Maize flour	China	26/27	14/27	13/27	11–68	80–3190	Ueno *et al*. (1997)
Sorghum	India	2/44	9/44	2/44	0.18–30.3	150–500	Vasanthi & Bhat (1998)
	Latin America						
Maize	Brazil, Venezuela	88/251	233/251	88/251	0.2–129	25–15 050	Medina-Martínez & Martínez (2000); Vargas *et al*. (2001)
	Brazil (total aflatoxins and total fumonisins)	17/150	147/150	17/150	38–460	96–22 000	Ono *et al*. (2001)

AFB_1, aflatoxin B_1; FB_1, fumonisin B_1

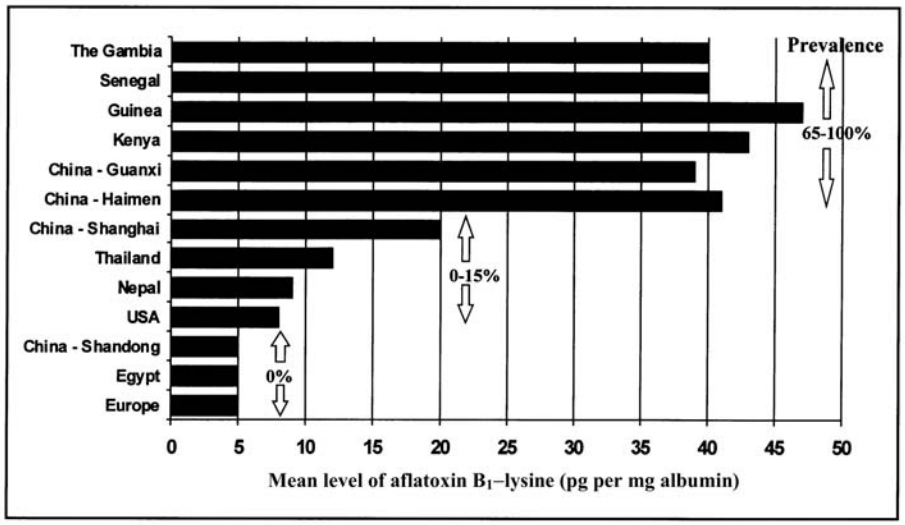
Table 7. Occurrence of aflatoxin M_1 in milk

Country	No. positive/ no. of samples	Range of aflatoxin M_1 concentrations (μg/kg)	Reference
Brazil	4/52	0.05–0.37	de Sylos *et al*. (1996); JECFA (2001)
Cuba	22/85	> 0.5	Margolles *et al*. (1992); Galvano *et al.* (1996)
Cyprus	11/112	0.01–0.04	Ioannou-Kakouri *et al.* (1999)
France	5284/5489 200/5489 5/5489	< 0.05 0.05–0.5 > 0.5	Dragacci & Frémy (1993); Castegnaro & Pfohl-Leszkowicz (1999)
	0/562	–	Castegnaro & Pfohl-Leszkowicz (1999)
Greece	3/81	0.05–0.18	Markaki & Melissari (1997)
India	89/504	0.1–3.5	Rajan *et al*. (1995)
Italy	122/214	0.003–0.101	Bagni *et al*. (1993); Galvano *et al.* (1996)
Japan	0/37	–	Tabata *et al*. (1993); Galvano *et al.* (1996)
Korea (Republic of)	50/134	0.05–0.28	Kim *et al*. (2000); JECFA (2001)
Spain	29/155	0.015–0.04	Jalon *et al*. (1994); Galvano *et al.* (1996)
Thailand	58/310	0.5–6.6	Saitanu (1997); JECFA (2001)
Europe	314/7573	≤ 0.05	European Commission (1998a); JECFA (2001)

albumin. Experimental data have also shown that this biomarker reflects the formation of the reactive metabolite of aflatoxin B_1 and the level of DNA damage occurring in the livers of rats treated with aflatoxin B_1. Figure 2 shows data from a number of populations, with adduct levels expressed as picograms of aflatoxin B_1–lysine equivalents per milligram of serum albumin. Other measurements of aflatoxin–DNA and aflatoxin–protein adducts in humans are discussed in Sections 4.4 and 4.5.

Maxwell (1998) has discussed the presence of aflatoxins in human body fluids and tissues in relation to child health in the tropics. In Ghana, Kenya, Nigeria and Sierra

Figure 2. Level and prevalence of aflatoxin exposure



From Montesano *et al.* (1997)
Data are expressed as pg aflatoxin B_1–lysine equivalents/mg serum albumin and represent the mean levels in samples with levels above the detection limit of the enzyme-linked immunosorbent assay used (5 pg/mg). Shandong (China), Europe (France and Poland) and Egypt are represented at the detection limit, but no samples were above this level of adduct (0% prevalence). The number of sera analysed varies per country. Original data are from Wild *et al.* (1993a) and Yu (1995).

Leone, 25% of cord blood samples contained aflatoxins, primarily M_1 and M_2, as well as others in variable amounts (range: 1 ng aflatoxin M_1/L to 64 973 ng aflatoxin B_1/L).

Of 35 cord serum samples from Thailand, 48% contained aflatoxins at concentrations of 0.064–13.6 nmol/mL (mean, 3.1 nmol/mL). By comparison, only two of 35 maternal sera obtained immediately after birth contained aflatoxin (mean, 0.62 nmol/mL). These results show that transplacental transfer and concentration of aflatoxin by the feto-placental unit occur (Denning *et al*., 1990).

Analyses of breast milk in Ghana, Nigeria, Sierra Leone and Sudan showed primarily aflatoxin M_1, aflatoxin M_2 and aflatoxicol. Aflatoxin exposure pre- or post-natally at levels ≥ 100 ng/L was very often associated with illness in the child (Maxwell, 1998).

Exposure of infants to aflatoxin M_1 from mothers' breast milk in the United Arab Emirates has been measured by Saad *et al.* (1995). Among 445 donors of breast milk, 99.5% of samples contained aflatoxin M_1 at concentrations ranging from 2–3 μg/L. The mothers were of a wide range of nationalities, ages and health status; no correlation was observed between these factors and aflatoxin M_1 content of the milk.

El-Nazami *et al.* (1995) measured levels of aflatoxin M_1 in breast milk in 73 women from Victoria, Australia and 11 women from Thailand. Aflatoxin M_1 was detected in 11 samples from Victoria (median concentration, 0.071 μg/L) and five samples from

Thailand (median concentration, 0.664 μg/L). Levels were significantly higher in the Thai samples.

1.3.5 *Occupational exposure to aflatoxins*

Kussak *et al*. (1995) demonstrated the presence of aflatoxins in airborne dust from feed factories.

During unloading of ships, aflatoxin B_1 has been found in bilge at levels as high as 300 ng/m^3 (Lafontaine *et al.*, 1994).

Autrup *et al*. (1993) assessed the exposure to aflatoxin B_1 of workers in animal feed processing plants in Denmark. The workers served as their own controls; blood samples were taken after their return from vacation and after four weeks of work. Binding of aflatoxin B_1 to serum albumin was measured. Seven of 45 samples were positive for aflatoxin B_1 with an average daily intake of 64 ng/kg bw aflatoxin B_1. The exposed workers had been unloading cargoes contaminated with aflatoxin B_1 or working at places where the dust contained detectable amounts of aflatoxin B_1. This level of exposure could partly explain the increased risk for liver cancer in workers in the animal feed processing industry.

Ghosh *et al*. (1997) assayed airborne aflatoxin in rice- and maize-processing plants in India using an indirect, competitive enzyme-linked immunosorbent assay. Levels of airborne aflatoxin were always higher in the respirable dust samples (< 7 μm) than in total dust samples. Concentrations of total airborne aflatoxin in the respirable dusts in the rice mill were 26 pg/m^3 and 19 pg/m^3 in the workplace and the storage area, respectively. Airborne aflatoxin was not detected in control sites of either of the grain-processing plants or in total dust samples obtained from the maize plant. At three sites in the maize-processing plant — the elevator (18 pg/m^3), the loading/unloading area (800 pg/m^3) and the oil mill (816 pg/m^3) — airborne aflatoxin was present only in the respirable dust samples.

In a study of factories in Thailand (Nuntharatanapong *et al*., 2001), samples of airborne dust generated during handling of animal feed were analysed in order to assess worker exposure to aflatoxins. The average aflatoxin level in the control air samples was 0.99 ng/m^3. Higher levels of aflatoxins were found in the air samples taken by samplers carried by five workers adding hydrated sodium calcium aluminosilicate to animal feed (1.55 ng/m^3) and five workers adding glucomannan, a viscous polysaccharide, to animal feed (6.25 ng/m^3). The exposed workers had altered lactate dehydrogenase isoenzyme activity and tumour necrosis factor levels in plasma. These changes may be associated with inhalation of mycotoxins and other contaminants in foodstuffs.

1.4 International exposure estimates

1.4.1 *JECFA (1998)*

In 1997, the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) performed an exposure estimate for aflatoxins (JECFA, 1998). This report summarized the results of monitoring and available national estimates of intake of aflatoxins in order to provide a framework for estimating increments in intake of aflatoxins. Estimates were based on the results of available monitoring data. Total intake of aflatoxins based on the GEMS (Global Environment Monitoring System)/Food Regional Diets (WHO, 1998) was used to evaluate the impact of four different scenarios: no regulatory limit and limits set at 20, 15 and 10 μg/kg aflatoxins. The evaluation was carried out for total aflatoxins and aflatoxin B_1 in maize and peanuts. The data submitted were not considered to be representative because sampling largely focused on those lots that were most likely to contain the highest levels of aflatoxin. However, JECFA considered the analysis to provide useful qualitative comparisons between regulatory options.

JECFA received data for this analysis from at least one country on every continent. The submitters generally considered the data to be biased towards the upper end of intake. In some cases, JECFA required individual data points in order to generate distributions and to evaluate the impact of imposing upper limits on aflatoxin in foodstuffs. Hence, data reported by the USA, China and Europe were used because the raw data were available. The reader is referred to the original report for a more complete discussion of the data.

JECFA used three pieces of information to estimate the potential intakes due to aflatoxin in imported crops: (*a*) levels of aflatoxin in imported crops; (*b*) the amounts of each imported crop consumed; and (*c*) the effect of any subsequent processing on aflatoxin levels. It then applied methods for combining these three factors to estimate intake.

(*a*) *Aflatoxin levels in foods: general*

The 1995 compendium, *Worldwide Regulations for Mycotoxins* (FAO, 1997) summarized reports from 90 countries. The data submitted by 33 countries for aflatoxin B_1 and 48 countries for total aflatoxins (B_1, B_2, G_1 and G_2) were used to estimate median levels of 4 and 8 μg/kg, respectively, in foodstuffs. The range of levels reported for aflatoxin B_1 was from 0 to 30 μg/kg and for total aflatoxins from 0 to 50 μg/kg. Seventeen countries provided information on aflatoxin M_1 in milk, with a median of 0.05 μg/kg and a range of 0–1 μg/kg.

Participants in the European Union Scientific Cooperation (EU SCOOP) assessment of aflatoxin (SCOOP, 1996) reviewed data submitted by member countries and by Norway. JECFA concluded that the results were unlikely to be representative and should not be used to estimate total aflatoxin intake for individual countries or for Europe. However, some insights were gained. SCOOP concluded that aflatoxins were found in a

broader range of foods than had been previously assumed, but that most samples did not contain any detectable aflatoxin. Sampling methods were very important in estimating aflatoxin levels accurately. In addition, different methods of collecting food consumption data may have made a difference in estimating aflatoxin intakes.

(*b*) *National estimates of aflatoxin intake (from JECFA, 1998)*

(i) *Australia*

From Australian market basket surveys, intake was estimated for average and extreme consumers. The average diet was estimated to give an intake of 0.15 ng aflatoxin per kg body weight per day and the upper 95th percentile diet approximately twice that level. Children's diets were estimated to give an intake up to approximately 0.45 ng/kg bw per day for the 95th percentile two-year-old (National Food Authority, 1992, reported by JECFA, 1998).

(ii) *China*

A series of intake and market basket studies have been conducted in China since 1980 to estimate the aflatoxin B_1 intakes, which were reported to range from 0 to 91 µg/kg bw per day (Chen, 1997).

(iii) *European Union*

Nine countries provided estimates of aflatoxin intake to the EU SCOOP project. None of these estimates was considered to be representative and all were viewed only as indicators of intake of aflatoxin. These estimates ranged from 2 to 77 ng per person per day for aflatoxin B_1 and from 0.4 to 6 ng per person per day for aflatoxin M_1. JECFA noted that these levels should not be used as estimates of intake either for a particular country or for Europe (JECFA, 1998).

(iv) *USA*

The US Food and Drug Administration (FDA) estimated intakes using data from the National Compliance Program for maize, peanut and milk products using Monte Carlo simulation procedures and data from the 1980s. Results differed only slightly from those of a repeat analysis in 1992 (Henry *et al.*, 1997). The lifetime intake of total aflatoxin was 18 ng per person per day for consumers only; intake for the 90th percentile individuals was 40 ng per person per day. For aflatoxin M_1, mean intake was 44 ng per person per day and for the 90th percentile individual 87 ng per person per day. Many assumptions were made in these estimates which would tend to bias the results upward.

(v) *Zimbabwe*

The theoretical maximum intake of aflatoxin M_1 for a child's diet containing 150 g maize with 5 µg/kg aflatoxin B_1 and 30 g peanuts with 10 µg/kg aflatoxin B_1 was estimated to be 1.05 µg per day (JECFA, 1998).

(*c*) *Impact of establishing maximum limits on estimate of intake*

Data from the EU, China and the USA were used to assess the potential impact of successfully eliminating aflatoxin levels above 20 μg/kg versus 15 μg/kg versus 10 μg/kg versus no limit for maize and peanuts (JECFA, 1998). The reader is referred to the full report for the tables, which more fully describe these four scenarios. JECFA emphasized that the aflatoxin levels presented in this report were not considered to be representative of the food supply in any country or of the commodities moving in international trade. The lack of representative data severely limited the ability to make quantitative estimates of aflatoxin intake; in general, the results were considered to be biased upwards. The data did provide, as JECFA stated, sufficient information to evaluate the likely impact of limiting aflatoxin levels in foodstuffs. Of the scenarios considered, the greatest relative impact on estimated average aflatoxin levels was achieved by limiting aflatoxin contamination to less than 20 μg/kg, i.e., eliminating all samples above 20 μg/kg from the food supply. Only small incremental reductions could be achieved by limiting aflatoxin levels to no more than 15 or 10 μg/kg.

1.4.2 *JECFA 2001*

In February 2001, at the request of the Codex Committee on Food Additives and Contaminants (Codex Alimentarius, 2000), JECFA (2001) evaluated the human health risks associated with consumption of milk contaminated with aflatoxin M_1 at two maximum regulatory levels of 0.05 μg/kg and 0.5 μg/kg. This project involved estimating exposure to aflatoxin M_1 in consumers from countries all over the world consuming milk and milk products.

Data on aflatoxin M_1 contamination of milk and/or milk products were submitted from Argentina, Canada, the Dubai Municipality of the United Arab Emirates, the EU, Indonesia, Korea (Republic of), Norway, the Philippines, Thailand and the USA. The majority of samples were submitted from the USA and the European Commission; very few were from south-east Asia and none from Africa. Of 6181 samples submitted from the USA and collected in south-western and southern states between 1998 and 2000, 1392 had aflatoxin M_1 levels between 0.05 and 0.5 μg/kg, and 113 had levels > 0.5 μg/kg. However, no samples were available from the north-eastern USA, where aflatoxin rarely occurs; most samples came from south-eastern parts where aflatoxin contamination often occurs in maize and peanuts consumed by dairy cows.

The data submitted by the European Commission represented 7573 samples from Austria, Belgium, Finland, France, Germany, Ireland, the Netherlands, Portugal, Sweden and the United Kingdom collected in 1999; 96% of the samples had aflatoxin M_1 levels below the limit of detection (which varied between countries: 0.001–0.03 μg/kg). The concentration in samples where aflatoxin M_1 was detected were ≤ 0.05 μg/kg (JECFA, 2001).

(*a*) *Estimates of aflatoxin M_1 intake using GEMS (Global Environment Monitoring System)/Food Regional Diets*

The GEMS/Food Regional Diets (WHO, 1998) are tables of dietary intakes of food commodities for five geographical areas. The major food class responsible for aflatoxin M_1 intake was identified as milk. The term 'milk' was assumed to include the mammalian milks (buffalo, camel, cattle, goat and sheep) listed in the GEMS/Food Regional Diets, but not to include cheese, butter or other dairy products derived from milk. In Table 8, exposure to aflatoxin M_1 from milk was calculated using three concentrations for the five geographical areas. [The use of dietary data to estimate mycotoxin intake can be misleading. Local climatic and other factors can greatly influence levels of mycotoxins in foods.] The first concentration was 0.05 μg/kg (the proposed maximum limit), the second was 0.5 μg/kg (the current maximum limit) and the third was the weighted mean of values for the geographical area. The use of 0.5 μg/kg as the level of aflatoxin M_1 in milk probably encompasses most of the milk samples and overestimates exposure. JECFA (2001)

Table 8. Estimated potential daily exposure to aflatoxin M_1 from all milks in five regional diets

Region/exposure	Aflatoxin M_1 in milk (μg/kg)	Aflatoxin M_1 intake (ng/person/day)
Europe/USA/Canada (0.294 kg milk/day)		
Proposed ML	0.05	14.7[a]
Current ML	0.5	147.0[a]
Weighted mean	0.023	6.8
Latin America (0.160 kg milk/day)		
Proposed ML	0.05	8.0[a]
Current ML	0.5	80.0[a]
Weighted mean	0.022	3.5
Far East (0.032 kg milk/day)		
Proposed ML	0.05	1.6[a]
Current ML	0.5	16.0[a]
Weighted mean	0.36	12
Middle East (0.116 kg milk/day)		
Proposed ML	0.05	5.8[a]
Current ML	0.5	58.0[a]
Weighted mean	0.005	0.6
Africa (0.042 kg milk/day)		
Proposed ML	0.05	2.9[a]
Current ML	0.5	20.9[a]
Weighted mean	0.002	0.1

From JECFA (2001)
ML, maximum level
[a] Calculated by the Working Group

used weighted means (including samples with zero values or values less than the limit of detection or quantification) to estimate dietary exposures for aflatoxin M_1. Because there were many non-detectable levels of aflatoxin M_1 in milk from the various studies and reports, the use of weighted means of all values could underestimate exposure for those individuals who are routinely exposed to higher levels of aflatoxin M_1 from milk.

(*b*) *Limitations of exposure estimates*

(i) The data submitted to FAO/WHO may not have been representative of countries or geographical areas, and not all member countries submitted data.

(ii) There were difficulties in attempting to compare and aggregate data on aflatoxin M_1 levels from different laboratories because the laboratories used different analytical methods. Also the data were presented in different ways (distributions, means of positive values, values less than a maximum limit).

(iii) The use of different analytical methods (thin-layer chromatography (TLC), HPLC) probably affected reported concentrations of aflatoxin M_1 in milk and therefore may affect intake estimates. Some methods, such as TLC, are more sensitive than others.

(iv) It was not possible to ascertain the effects of processing, season, climate or other environmental variables on the aflatoxin M_1 content of milk. These effects were often not addressed by the various studies and reports, and different descriptors were used for milk and other dairy products (e.g., 'raw' versus 'pasteurized') (JECFA, 2001).

1.4.3 *Exposure to aflatoxin M_1 in the French population*

Verger *et al*. (1999) have estimated exposures to aflatoxin M_1 in the French population (Table 9).

Table 9. Estimated average intake of aflatoxin M_1 in France

Type of product	Aflatoxin content (μg/kg)	ng/day per kg body weight				
		Mean	SD	95th percentile	Average % in the total intake	95th percentile/ mean
Milk and extra fresh milk[a]	0.014	[0.048]	0.107	0.261	59.2%	3.1
Cheeses[b]	0.093	0.058	0.050	0.143	40.8%	2.5
Total	–	0.142	0.122	0.362	100%	2.6

From Verger *et al.* (1999)

[a] Aflatoxin content of milk and extra fresh milk calculated from Direction Générale de l'Alimentation (DGAL), Paris (1995)

[b] Aflatoxin content of cheeses calculated from DGAL (1995) using a conversion coefficient from milk to cheese of 6.5

1.5 Regulations and guidelines

Efforts to reduce human and animal exposure to aflatoxins have resulted in the establishment of regulatory limits and monitoring programme worldwide. The rationale for the establishment of specific regulations varies widely; however, most regulations are based on some form of risk analysis including the availability of toxicological data, information on susceptible commodities, sampling and analytical capabilities, and the effect on the availability of an adequate food supply (Stoloff *et al.*, 1991). In 1995, among countries with more than five million inhabitants, 77 had known regulations for mycotoxins (all of which included aflatoxins) and 13 reported the absence of regulations. Data were not available for 40 countries (FAO, 1997). The regulation ranges for aflatoxin B_1 and total aflatoxins (B_1, B_2, G_1, G_2) were 'none detectable' to 30 or 50 μg/kg, respectively. Seventeen countries had regulations for aflatoxin M_1 in milk. The regulatory range for aflatoxin M_1 in milk was 'none detectable' to 1.0 μg/kg. New minimum EU regulations to which all EU countries must adhere were provided in 1998 (European Commission, 1998b). These regulations apply to all aflatoxins (B_1, B_2, G_1, G_2) in raw commodities and processed foods and to aflatoxin M_1 in milk. Regulations for other commodities include infant foods (European Commission, 2001) and selected spices (European Commission, 2002).

The Codex Alimentarius Commission (1999) is considering a recommendation to establish a limit for aflatoxins in foods of 15 μg/kg of total aflatoxins for all foods worldwide.

2. Studies of Cancer in Humans

Beginning in the 1960s and throughout the 1980s, a large number of ecological correlation studies were carried out to look for a possible correlation between dietary intake of aflatoxins and risk of primary liver cancer (IARC, 1993). Most of these studies were carried out in developing countries of sub-Saharan Africa or Asia, where liver cancer is common. With some notable exceptions, and despite the methodological limitations of these studies, they tended to show that areas with the highest presumed aflatoxin intake also had the highest liver cancer rates. However, the limitations of these studies, including questionable diagnosis and registration of liver cancer in the areas studied, questionable assessment of aflatoxin intake at the individual level, non-existent or questionable control for the effect of hepatitis virus and the usual problem of making inferences for individuals from observations on units at the ecological level, led to increasing recognition of the need for studies based on individuals as units of observation.

In the 1980s, some case–control studies were carried out in high-risk areas, generally based on reasonably reliable diagnostic criteria for liver cancer (IARC, 1993). The comparability of cases and controls was limited in some of these studies. Exposure to

aflatoxins was sometimes assessed via dietary questionnaires and sometimes via biomarker measurements. As both of these were collected after disease onset, their relevance to past lifetime intake of aflatoxins was uncertain. Beginning in the mid 1980s, some prospective cohort studies were undertaken which avoided many of the methodological limitations of earlier studies. Among the major advantages of this new generation of studies were the following: new improved biomarkers of aflatoxin exposure, improved ability to measure hepatitis infection, better comparability of cases and controls within a well defined cohort, and control of the temporal sequence by measuring exposure before disease onset.

In 1992, an IARC Working Group described all relevant human studies that had been reported and concluded that there was *sufficient evidence* in humans for carcinogenicity of aflatoxin B_1 and of naturally-occurring mixtures of aflatoxins. The present monograph represents an update of evidence published since that evaluation was made, without describing the studies covered in the previous monograph, although brief summaries of the main studies are given in tabular format (Tables 10–12). These tables also provide summaries of the relevant studies that have been published since 1993 and which are described in the following sections. The outcome investigated in most studies was liver cancer. Different studies used different sources (e.g., death certificates, hospital registries, medical examinations) and different criteria (clinical, cytological) for definition of liver cancer. Different terms, such as liver cancer, primary liver cancer or hepatocellular carcinoma (HCC) were used. In the following descriptions, we have used the terminology used by the authors.

2.1 **Descriptive studies** (see Table 10)

Hatch *et al*. (1993) conducted a hybrid ecological cross-sectional study in eight areas of Taiwan (China), with a wide range of rates of mortality from primary hepatocellular carcinoma (HCC). In order to derive estimates of aflatoxin levels in the eight areas, they selected a representative sample of 250 adult residents in total (unequal numbers per area). Participants were interviewed and were asked to provide both morning urine and blood specimens. Serum was used for detecting hepatitis B surface antigen (HBsAg). Urine was used for detecting aflatoxins B_1 and G_1 and metabolites, including aflatoxins M_1 and P_1; the highest sensitivity was for aflatoxin B_1. Measured values ranged from 0.7 to 511.7 pg equivalents of aflatoxin B_1/mL of urine, with a mean of 41.3 pg/mL. Mean levels were similar in men and women, and in hepatitis B virus (HBV) carriers and HBV non-carriers. The primary analyses were carried out with individuals as the unit of analysis. In these analyses, the individual's measurements of aflatoxin B_1 equivalents and of HBsAg were used in conjunction with the HCC rate (sex-specific, age-adjusted) of the entire area in which the individual resided. There were 246 data points for these analyses (four individuals had missing blood specimens). Some bivariate correlation coefficients and some regression analyses in which aflatoxin levels were regressed on area HCC mortality, HBsAg, age and sex were calculated. In addition, in some analyses the data

Table 10. Summary of the principal ecological and cross-sectional studies on liver cancer and aflatoxins

Reference	Area	Units of observation/ number of units	Exposure measure(s)	Outcome measure(s)	Covariate	Results	Comments
Alpert *et al.* (1971)	Uganda	Main tribes and districts of Uganda; 7	Aflatoxin contamination of nearly 500 food samples taken from randomly selected native homes and markets; 1966–67	Hepatoma incidence identified from hospital records; 1963–66	Nil	The highest incidence of hepatoma occurred in areas with highest levels of aflatoxin contamination.	
Peers & Linsell (1973)	Kenya	Altitude areas of Murang'a district; 3	Aflatoxin extracted from food samples, repeated cluster sampling over 21 months	Incident hepatocellular cancers ascertained from local hospitals; 1967–70	Nil	Using 6 data points (3 areas, both sexes), correlation ($r = 0.87$) between aflatoxin intake and liver cancer	Questionable completeness of liver cancer registration. Small number of units of observation
Peers *et al.* (1976)	Swaziland	Altitude areas; 4	Aflatoxin from food and beer samples: every 2 months for 1 year, over 1000 samples analysed; 1972–73	PLC incidence rates, from national cancer registry; 1964–68	Nil	Correlation (males, $r = 0.99$; females, $r = 0.96$) between aflatoxin intake and PLC rates	Exposure post-dated cancer data
Wang *et al.* (1983)	China	29 provinces and municipalities; 552 cities	Grain oil contamination by aflatoxin B_1	PLC mortality	HBsAg, climate	Contamination by aflatoxin strongly correlated with liver cancer	Incomplete study description
Stoloff (1983)	USA	South-east, north and west regions of USA	Daily aflatoxin ingestion among males, based on historic food consumption surveys and historic estimates of aflatoxin contamination	PLC; 1968–71 and 1973–76	Nil	South-east had much higher aflatoxin ingestion and 10% higher PLC rates than 'north and west'	Considerable excess of PLC observed among Orientals and urban black males
Van Rensburg *et al.* (1985)	Southern Africa	7 districts of Mozambique; Transkei, South Africa	Mean aflatoxin contamination of food samples, over 2500 samples analysed; 1969–74	Mozambique: incidence rates of HCC; 1968–75 (variety of sources including local hospitals and South African mines); Transkei; 1965–69	Nil	Rank correlations between HCC and mean total aflatoxin 0.64 ($p < 0.05$) in men and 0.71 ($p < 0.01$) in women	

Table 10 (contd)

Reference	Area	Units of observation/ number of units	Exposure measure(s)	Outcome measure(s)	Covariate	Results	Comments
Autrup *et al*. (1987)	Kenya	Districts of Kenya; 9	Urinary 8,9-dihydro-8-(7-guanyl)-9-hydroxy-AFB_1 as ascertained in surveys at outpatient clinics in the 9 districts (total sample, 983); 1981–84	Primary hepatocellular carcinoma (PHC) incidence diagnosed at one large hospital in Nairobi; 1978–82	HBsAg and anti-HBc	*Spearman rank correlation* (r) with PHC rate Prevalence of AFB_1 0.75 HBV 0.19	Potential confounding by ethnicity. No interaction between AFB_1 and HBV
Peers *et al*. (1987)	Swaziland	Topographic (4) and administrative regions (10)	Aflatoxins measured in food samples from households and crop samples from fields; over 2500 samples analysed; 1982–83	Incidence rates of PLC; 1979–83	HBsAg and other markers of HBV infection in 3047 serum samples from the Swaziland blood bank	Significant correlation between estimated aflatoxin (and AFB_1) consumption and PLC; little effect of HBsAg on PLC	
Campbell *et al*. (1990)	China	48 widely scattered counties – out of a total of 2392 in China	Mean urinary aflatoxin metabolites, serum HBsAg and 3-day dietary intake, based on local sample surveys in 1983	PLC mortality rates; 1973–75	HBsAg (50 individuals per country), alcohol, some others	Urinary aflatoxin and PLC (r = –0.17)	Positive associations between liver cancer and HBsAg (r = 0.45), liquor intake (r = 0.46), dietary cadmium (r = 0.40), plasma cholesterol (r = 0.42). Exposure data post-dated cancer data
Van Rensburg *et al*. (1990)	South Africa	Districts of the Transkei; 4	AFB_1 contamination of local food samples, based on over 600 samples; 1976–77	PLC incidence in residents and in gold-miners	Nil	Rank order correlations between AFB_1 intake and PLC incidence in goldminers from the Transkei were significant at $p < 0.05$.	

Table 10 (contd)

Reference	Area	Units of observation/ number of units	Exposure measure(s)	Outcome measure(s)	Covariate	Results	Comments
Srivatanakul *et al.* (1991a)	Thailand	Selected areas of Thailand; 5	Surveys of local residents, aflatoxin measured in urine and in serum, 50–100 subjects per area	Incidence rates of HCC and cholangio-carcinoma 1980–82. Standardized proportionate incidence ratio (PIR)	Same 100–200 individuals per area as for aflatoxin, HBsAg, anti-HBs, anti-HBc and liver fluke (OV)	HCC / Cholangiocarcinoma Serum aflatoxin: –0.75 (p = 0.14) / –0.03 (p = 0.96) Urinary aflatoxin: –0.64 (p = 0.25) / 0.17 (p = 0.78) Anti-OV titre: –0.37 (p = 0.54) / 0.98 (p = 0.004) HBsAg: –0.45 (p = 0.44) / 0.27 (p = 0.66)	
Hatch *et al.* (1993)	Taiwan	Townships; 8	Mean urine levels of various aflatoxins. Measured on a total of 250 randomly selected subjects in 8 townships	HCC mortality rate in the area of the township	HBsAg; smoking status, alcohol consumption	Univariate correlation between mean urinary aflatoxin and area HCC rates: men, 0.83 (p = 0.012) women, 0.49 (p = 0.22)	Univariate correlations between area HCC rates and mean HBsAg were around 0.50. When individuals were used as units of observation, with the area mortality rate attributed to the individual, the correlations between urinary aflatoxins and HCC were considerably attenuated.
Omer *et al.* (1998)	Sudan	Two areas, one high- risk, one low-risk	Peanut butter samples collected in markets and analysed for AFB_1. Type of storage assessed	Fragmentary data indicate that risk of liver cancer is higher in one area than the other.	–	Aflatoxin consumption levels were higher in the presumed high-risk area than in the presumed low-risk area.	Only two areas compared. Unreliable measures of liver cancer incidence

AFB_1, aflatoxin B_1; anti-HBc, antibody to hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; OV, *Opisthorchis viverrini*; PHC, primary hepatocellular carcinoma; PLC, primary liver cancer

were collapsed into a conventional ecological study with eight units of observation and the mean levels of aflatoxin and HBsAg correlated with HCC rates. The univariate correlations between HCC and aflatoxins at the ecological level were 0.83 ($p = 0.01$) in men and 0.49 ($p = 0.22$) in women. The correlations were much lower, albeit statistically significant, when analysed at the individual level: 0.29 ($p = 0.002$) in men and 0.17 ($p = 0.047$) in women. In the multivariate regression analysis, HCC was significantly associated with aflatoxin levels, after adjusting for age, sex and HBsAg. Adjustments for smoking and alcohol, in a subset of 190 subjects with available interview data, and the inclusion of interaction terms did not materially affect the findings. Thus, the very different types of analysis all pointed to an association between urinary aflatoxins and HCC. [The Working Group noted that the calculation of the *p* value did not take into account the clustered sampling design.]

Omer *et al*. (1998) carried out a comparison of aflatoxin contamination of peanut products in two areas of Sudan. On the basis of 'clinical experience and Khartoum hospital records', the authors suspected that incidence of HCC was substantially higher in western Sudan than in central Sudan. The study was carried out in 1995 and involved selection of peanut butter samples from local markets using a staged sampling approach to identify markets in the two study areas. Samples were characterized as to how they had been stored and were analysed for aflatoxin B_1 by HPLC. Mean aflatoxin B_1 levels were much higher in 'high-risk' western Sudan (87.4 ± 197.3 μg/kg) than in central Sudan (8.5 ± 6.8 μg/kg). Also, dietary questionnaires among subjects recruited for a small case–control study indicated that residents in western Sudan consumed more peanut butter than residents in central Sudan. [The Working Group noted that only two areas were compared, and that there was no documented evidence of differences in the incidence rates of HCC between the two areas. A small case–control study was carried out as well, but the Working Group noted that this was probably a small feasibility study that was superseded by Omer *et al*. (2001).]

2.2 **Cohort studies** (see Table 11)

Qian *et al*. (1994) updated a cohort study previously described by Ross *et al*. (1992) of 18 244 male residents of Shanghai, China, 96% of whom were aged 45–64 years on entry to the study. The men were recruited by invitation from four geographically defined areas and responded to questionnaires administered by nurses, usually in their homes, on lifestyle (including smoking and alcohol consumption) and on food frequency. Blood and urine specimens were collected. The men were followed up by identification of death records in district vital statistics units and through linkage with the Shanghai Cancer Registry (estimated to be 85% complete). An attempt was also made to contact each cohort member annually. The cohort was established between January 1986 and September 1989 and was followed to 1 February 1992 for the current analysis, resulting in 69 393 person–years of follow-up. Of 364 cancer cases identified, 55 were diagnosed as primary liver cancers, nine of which were confirmed by biopsy. The reported diet

Table 11. Summary of the principal cohort and nested case–control studies on liver cancer and aflatoxins

Reference	Area	Study base	Type of analysis	Exposure measures	Outcome measures	Covariate	Results	Comments
Hayes *et al.* (1984)	Netherlands	71 male oil-press workers exposed to dust containing aflatoxin	Cohort – SMR comparisons with Dutch males	Worked > 2 years in exposed area during 1961–70	Cancer mortality	–	Cases SMR 95% CI Lung cancer 7 2.5 1.0–5.0 Liver cancer 0 0 NA	
Yeh *et al.* (1985)	China	Selected villages in Fusui county, Guangxi	Cohort (ecological exposure)	Village mean for intake of aflatoxin based on food samples. ~10-fold difference between low and high contamination areas	Liver cancer mortality	HBsAg measured among cases	Aflatoxin contamination / HBsAg status / HCC deaths No. / HCC deaths Rate per 1000 p–yr Heavy Positive 13 649 Heavy Negative 2 99 Light Positive 1 66 Light Negative 0 0	Incomplete study description [unit presumed to be: per 1000 p–yr]
Olsen *et al.* (1988)	Denmark	Male employees of 241 livestock feed companies, employed after 1964	Cohort SPIR	Longest-held job (> 1964) in one of 241 companies	Liver cancer incidence traced in Danish Cancer Registry; 1970–84		Longest employment Cases SPIR 95% CI Ever 6 1.4 0.57–2.9 ≥ 10 yrs before diagnosis 7 2.5 1.1–4.9	Confounding by HBV and alcohol unlikely
Yeh *et al.* (1989)	China	Five communities of southern Guangxi Province, men enrolled in 1982–83; $n > 7917$	Cohort – PHC mortality rates in different categories of estimated mean AFB_1 consumption	Mean AFB_1 level of community of residence, as estimated from food samples collected from all over the region; 1978–84	Mortality from PHC based on follow-up 1984–86	HBsAg in cases and subcohort	Strong correlation between PHC mortality and estimated levels of AFB_1 in 4 communities; aflatoxin levels not available in one community (Pearson correlation coefficient, 1.00; $p = 0.004$)	Strong association observed between HBsAg and PHC mortality in cohort and nested case–control analysis (RR = 32); not seen in the ecological analysis ($r = 0.28$; $p = 0.65$)

Table 11 (contd)

Reference	Area	Study base	Type of analysis	Exposure measures	Outcome measures	Covariate	Results				Comments
Qian *et al.* (1994); Ross *et al.* (1992)	Shanghai, China	Men, mainly 45–64 years old; *n* = 18 244. Resident in one of four areas. Recruited during 1986–89	Both cohort analysis and a nested case–control analysis with 50 cases and 267 matched controls	Detailed dietary history linked to measured levels in sample foods	Liver cancer mortality and incidence follow-up to 1992. Intensive tracing	Measured HBsAg, cigarette smoking	Aflatoxin	Cases	RR	95% CI	Cohort analysis
							Medium	25	1.6	0.8–3.1	
							Heavy	16	0.9	0.4–1.9	
								Cases	OR	95% CI	Nested case–control analysis
							Aflatoxin–guanine adducts	18	9.1	2.9–29	
				Measured urinary aflatoxin and aflatoxin–guanine adducts			Any biomarker of aflatoxin	36	5.0	2.1–12	
							Joint exposure to any biomarker of aflatoxin and HBsAg	23	59	17–212	
Chen *et al.* (1996)	Taiwan, China	Penghu Islets. Over 6000 subjects enrolled. Possibly a subset of the cohort of Wang *et al.* (1996)	Nested case–control analysis based on 20 cases and 86 controls	Measured AFB_1–albumin adducts	HCC, ascertained by an active diagnostic procedure	Sociodemographic characteristics, HBsAg, anti-HCV, family history of HCC and liver cirrhosis		Cases	OR	95% CI	
							AFB_1–albumin adducts	13	5.5	1.2–25	
Wang *et al.* (1996a)	Taiwan, China	7 townships. Over 25 000 subjects enrolled in cohort	Nested case–control analysis based on 56 cases and 220 controls	Biomarker measurement of urinary aflatoxins and aflatoxin–albumin adducts	HCC, ascertained by a variety of tracing sources, 1991–95	Sociodemographic characteristics and HBsAg		Cases	OR	95% CI	*Adjusted for HBsAg
							Aflatoxin–albumin adducts	31	1.6*	0.4–5.5	
							Urinary aflatoxin	26	3.8*	1.1–13	
							Urinary aflatoxin + HBsAg	22	112	14–905	

Table 11 (contd)

Reference	Area	Study base	Type of analysis	Exposure measures	Outcome measures	Covariate	Results				Comments
Sun *et al.* (2001)	Taiwan, China	Same as Wang *et al.* (1996)	Nested case–control analysis based on 79 cases and 149 controls, all HBsAg-positive	Aflatoxin–albumin adducts	Same as Wang *et al.* (1996), 1991–97	Same as Wang *et al.* (1996), anti-HCV, $GSTM_1$, $GSTT_1$		Cases	OR	95% CI	Statistically significant interaction with $GSTT_1$ genotypes
							Aflatoxin–albumin adducts	47	2.0	1.1–3.7	
Yu *et al.* (1997a)	Taiwan	Male patients from Government Employee Central Clinics and a Taipei hospital, aged 30–65 during enrolment, 1988–92; *n* = 4841 HBsAg-positive, *n* = 2501 HBsAg-negative	Nested case–control on 43 cases and 2 matched controls per case, one HBsAg positive and one negative	Baseline interviews and measurement of urinary aflatoxin and aflatoxin adducts	HCC	Sociodemographic, alcohol, smoking		Cases	OR	95% CI	All analyses restricted to HBsAg-positive subjects
							AFM_1	23	6.0	1.2–29	
							AFP_1	18	2.0	0.5–8.0	
							AFB_1	17	2.0	0.7–5.8	
							AFB_1–*N*7-guanine adducts	6	2.8	0.6–13	
Lu *et al.* (1998)	China	Seven townships in Qidong. Men aged 20–60 years during enrolment; follow-up: 1987–97	Nested case–control among HBsAg carriers; 30 cases and 5 controls per case (matched for age, place of residence)	AFB_1–albumin adducts	PLC			Cases	OR	95% CI	
							AFB_1-albumin adducts	23	3.5	[1.3–10]	
Sun *et al.* (1999)	China	Men in 2 townships screened and found positive for chronic HBV infection, recruited in 1987–98; *n* = 145	Cohort analysis	AFM_1 measured in 8 pooled urinary samples	HCC	Anti-HCV, family history of HCC, smoking, alcohol		Cases	RR	95% CI	Increased risks for HCC among anti-HCV (RR, 6.0) and those with family history of HCC (RR, 4.7) *Adjusted for anti-HCV and HCC family history
							AFM_1	17	4.5*	1.6–13	

AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1; AFP_1, aflatoxin P_1; CI, confidence interval; GST, glutathione *S*-transferase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NA, not applicable; OR, odds ratio; OV, *Opisthorchis viverrini*; PHC, primary hepatocellular carcinoma; p–yr, person–years; RR, relative risk; SMR, standardized mortality ratio; SPIR, standardized proportionate incidence ratio

history based on a frequency checklist of 45 food items usually consumed as an adult was combined with a set of independently measured aflatoxin levels in various local foods to derive a quantitative measure of dietary aflatoxin exposure. In a cohort-type analysis, using the lowest tertile of aflatoxin exposure as reference, the middle tertile had a relative risk (adjusted for age and smoking) of 1.6 (95% confidence interval [CI], 0.8–3.1; 25 cases) and the highest tertile had an odds ratio (OR) of 0.9 (95% CI, 0.4–1.9; 16 cases).

To assess the risks in relation to biomarkers of aflatoxin exposure, a nested case–control study was conducted using 50 of the cases (Qian *et al.*, 1994). Controls were selected from among subjects who had no history of liver cancer on the date of cancer diagnosis of the index cases and were matched to cases in ratios ranging from 10:1 to 3:1, yielding a total of 267 controls. For each case and control, urine samples were analysed for aflatoxins B_1, P_1 and M_1 and for aflatoxin B_1–*N*7-guanine adducts, and among a subgroup of 28 cases and their matched controls for aflatoxins G_1 and Q_1. HBsAg was measured by radioimmunoassay. Thirty-two out of 50 cases and 31 out of 267 controls were HBsAg-positive. Each of the six biomarkers of aflatoxin exposure was more frequently present among cases than controls. For 36 of the 50 liver cancer cases and 109 of 267 controls, results were positive in at least one of the four assays analysed for the full set of cases and controls (adjusted relative risk, 5.0; 95% CI, 2.1–12). The highest risks were found among subjects with aflatoxin B_1–*N*7-guanine adducts. Compared with subjects who had no aflatoxin biomarkers and were HBsAg-negative, the interaction of the two factors was supra-multiplicative, with relative risks as follows: aflatoxin biomarker only, 3.4 (95% CI, 1.1–10); HBsAg only, 7.3 (95% CI, 2.2–24); both factors, 59 (95% CI, 17–212). [The Working Group noted inconsistencies between analyses based on dietary questionnaires and biomarkers.]

The Penghu Islets reportedly have the highest rates of HCC in Taiwan, China. Chen *et al*. (1996) enrolled 4691 men and 1796 women, aged 30–65 years, in a prospective cohort study. The subjects were selected from a housing register maintained by the local administration. Participants were interviewed on a variety of sociodemographic, dietary and medical history topics. Blood samples were collected and stored frozen. A two-stage screening process for HCC was undertaken which included serological markers and clinical assessments with ultrasonography. Subjects were further followed up with annual examinations. A total of 33 cases of HCC were diagnosed by December 1993, of whom two were negative for HBsAg. A total of 123 controls were selected from within the cohort among unaffected subjects, and matched with cases for age, sex, village and date of blood collection. Blood samples from cases and controls were analysed for HBsAg, for anti-hepatitis C virus (HCV) antibodies and aflatoxin B_1–albumin adducts, although samples for adduct analysis were usable for only 20 cases and 86 controls. Using logistic regression, with age and sex adjustment, and a detection limit for albumin adducts of 0.01 fmol/μg as the cut-off value, the OR for an association between presence of aflatoxin B_1–albumin adducts and HCC was 3.2 (13 cases; 95% CI, 1.1–8.9). When the statistical model also included several other covariates (HBsAg, anti-HCV, family history of liver cancer and cirrhosis), the odds ratio for aflatoxin B_1–albumin adducts

rose to 5.5 (95% CI, 1.2–25). There was also an extremely high risk associated with positive HBsAg status (OR = 129; 95% CI, 25–659). The authors surmised that peanut contamination was a major source of aflatoxin in this population.

Wang *et al*. (1996a) carried out a cohort study in seven townships of Taiwan, China, including three on the Penghu Islets and four on Taiwan Island. Of the total population of 89 342 eligible subjects selected from local housing offices and mailed an invitation in a cancer screening project, 25 618 (29%) volunteered to participate. Among participants, 47% were men and enrolment occurred from July 1990 to June 1992. Participants were interviewed to elicit information on sociodemographic characteristics, alcohol and smoking habits, and medical history. Fasting blood and spot urine specimens were collected and stored frozen. Serum samples were assayed for HBsAg and α-fetoprotein, anti-HCV and various markers of liver function. Abdominal ultrasonography was carried out among a subgroup of high-risk persons from two Penghu Islets. All participants were recontacted by invitation to local research centres or by telephone interviews between 1992–94. Periodic searches for death certificates from local housing offices and in June 1995 through linkage with the national death and cancer registries were carried out. The overall follow-up rate was > 98%. Between February 1991 and June 1995, 56 HCC cases were identified in the cohort, of which 22 were histologically/cytologically confirmed. For each case, four controls were selected among cohort members who were free of liver cancer or cirrhosis at the time of case identification, and who were matched for age, sex, township and recruitment date. Altogether there were 56 HCC cases and 220 controls. Serum and urine specimens were available for analysis on subsets: serum for 52 cases and 168 controls, and urine for 38 cases and 137 controls. Urinary aflatoxin metabolites were determined using a monoclonal antibody with high affinity to aflatoxin B_1 and significant cross-reactivity to aflatoxins B_2, M_1, G_1 and P_1. Serum aflatoxin–albumin adducts were measured. Using conditional logistic regression, the OR for liver cancer corresponding to detectable levels of aflatoxin–albumin adducts was 4.6 (95% CI, 2.0–10) before adjustment for HBsAg and 1.6 (95% CI, 0.4–5.5) after adjustment. [The Working Group noted inconsistencies in numbers of available controls for serum aflatoxin–albumin adducts.] For high levels of urinary aflatoxin metabolites, the OR was 3.3 (95% CI, 1.4–7.7) before adjustment for HBsAg and 3.8 (95% CI, 1.1–13) after adjustment. While there was little or no effect of aflatoxin biomarkers on HCC among HBsAg-negative subjects, there were quite strong effects among HBsAg-positive subjects, especially in the analysis using aflatoxin metabolites as the exposure. [It seems that the present cohort and the cases identified in it include the cases studied by Chen *et al*. (1996) in the Penghu Islets. The low participation rate would not have affected the validity of the results unless individuals with preclinical liver cancer symptoms and high aflatoxin exposure were more likely to volunteer for participation in the study than others in the same population.]

Sun *et al*. (2001) reported the results from a nested case–control study of an extended follow-up of the cohort described by Wang *et al*. (1996a). Seventy-nine HBsAg-positive cases of HCC were identified between 1991 and 1997, and matched for age, gender,

residence and date of recruitment to one or two randomly selected HBsAg-positive controls (total, 149). Blood samples were collected and analysed for HBV and HCV, for aflatoxin B_1–albumin adducts, and for glutathione *S*-transferase (GST) M1 and T1 genotypes. In a conditional logistic regression analysis, a significant relationship was observed between HCC risk and aflatoxin B_1–albumin adducts (OR = 2.0; 95% CI, 1.1–3.7). *GSTM1*- and *GSTT1*-null genotypes were associated with a decreased risk for HCC (OR = 0.4; 95% CI, 0.2–0.7 and OR = 0.5; 95% CI, 0.2–0.9). A statistically significant (p = 0.03) interaction was found between aflatoxin B_1–albumin adducts and *GSTT1* genotype, indicating a more pronounced risk among those who were *GSTT1*-null genotype (OR = 3.7; 95% CI, 1.5–9.3), and no risk among those who had the non-null genotype (OR = 0.9; 95% CI, 0.3–2.4).

Yu *et al*. (1997a) carried out a cohort study in Taiwan, China, to study the role of aflatoxin in the etiology of HCC. Between 1988 and 1992, a cohort of 4841 male asymptomatic HBsAg carriers and 2501 male non-carriers, aged 30–65 years, was recruited from the Government Employee Central Clinics and the Liver Unit of a hospital in Taipei. At entry into the study, each participant was interviewed to obtain information on demographic characteristics, habits of cigarette smoking and alcohol drinking, diet (including the frequency of consuming peanuts and fermented bean products, which are thought to be the major aflatoxin-contaminated foodstuffs in Taiwan), as well as personal and family history of major chronic diseases. Urine and blood samples from study subjects were stored frozen. All HBsAg carriers in this study had both ultrasonography and α-fetoprotein measurement every 6–12 months. Follow-up of HBsAg non-carriers was carried out by annual examination including a serum α-fetoprotein test. The response rate to the periodic follow-up examinations was approximately 72% for HBsAg carriers and 80% for HBsAg non-carriers. Information on HCC occurrence and vital status of study subjects who did not participate in the follow-up examinations was obtained from both computerized data files of the national death certification and the cancer registry. By 31 December 1994, 34 579 person-years of follow-up had been accumulated, an average of 4.7 years per person. Fifty HCC cases were identified during the follow-up period. All HCC cases were diagnosed on the basis of either pathological and cytological examinations or an elevated α-fetoprotein level combined with at least one positive image. To investigate the role of aflatoxin, a nested case–control comparison was carried out, in which two separate matched controls per case were selected, one who was HBsAg-positive and one who was HBsAg-negative. Levels of aflatoxin metabolites in urine were analysed by reverse-phase HPLC allowing measurement of aflatoxins M_1, P_1, B_1 and G_1 and aflatoxin B_1–*N*7-guanine. Most subjects were also tested for anti-HCV. After exclusion of subjects with missing specimens, analyses were available on 43 matched case–control sets. Among all HCC cases, only one occurred in the HBsAg-negative subcohort, and that one was positive for anti-HCV. All study subjects were positive for aflatoxin M_1, 81% for aflatoxin P_1, 43% for aflatoxin B_1–*N*7-guanine adducts, 28% for aflatoxin B_1 and 12% for aflatoxin G_1. There was a significant correlation (r = 0.35) between reported dietary intake of various foods thought to contain aflatoxins and levels of urinary

aflatoxin M_1. No significant correlations with other aflatoxin metabolites were observed. The main analyses, using conditional logistic regression, were carried out among cases and controls who were HBsAg carriers. Four of the five aflatoxin markers, but not aflatoxin G_1, were associated with an elevated risk for HCC among subjects in the highest tertile of exposure, although only for aflatoxin M_1 was this significant. The OR in the highest tertile of aflatoxin M_1 exposure, after adjustment for education, ethnicity, alcohol and smoking, was 6.0 (23 cases; 95% CI, 1.2–29). When pairs of these aflatoxin biomarkers were examined, certain combinations were found to be associated with particularly high risk: thus, subjects with detectable aflatoxin B_1– *N*7-guanine and high levels of aflatoxin M_1 had an OR of 12 (16 cases; 95% CI, 1.2–117).

To investigate the role of HBV and aflatoxin in the etiology of liver cancer, Lu *et al*. (1998) carried out a nested case–control analysis within a cohort of 737 male HBsAg carriers and 699 HBsAg non-carriers in Qidong, China (follow-up was from 1987–97). Among the HBsAg carriers, 30 cases of liver cancer were matched for age and place of residence with 150 non-cases from the cohort. Levels of aflatoxin B_1–albumin adducts were significantly higher among cases than among controls, both in proportion and in concentration. The crude OR was 3.5 [95% CI, 1.3–10].

Sun *et al*. (1999) reported on the experience of a cohort of 145 men with chronic hepatitis B. These HBV-positive men had been detected in a prevalence survey carried out in 1981–82 in two townships in Qidong, China. They were recruited for the present follow-up study during 1987–98. At recruitment, they were interviewed and examined; eight urine samples were obtained at monthly intervals and blood was drawn periodically throughout the follow-up period. The urine samples for each individual were pooled and aflatoxin M_1 was measured in the pooled sample. No patients were lost to follow-up. The mean age of the cohort was 39 years in 1998. Over the period of follow-up, 22 of the 145 subjects were diagnosed with liver cancer, of whom 10 had pathological confirmation. Anti-HCV-positive subjects had an increased risk for HCC compared with subjects who were anti-HCV-negative and subjects with a family history of HCC had an increased risk compared with subjects who did not have a family history of HCC. The median concentration of aflatoxin M_1 in urine was 9.6 ng/L and the highest concentration was 243 ng/L. Using 3.6 ng/L as the cut-point in a Cox proportional hazard model, the relative risk for HCC among subjects with high aflatoxin M_1 compared with those having low aflatoxin M_1 was 3.3 (95% CI, 1.2–8.9). When anti-HCV status and family history of HCC were also included in the model, the relative risk for HCC associated with aflatoxin M_1 was 4.5 (95% CI, 1.6–13).

2.3 **Case–control studies** (see Table 12)

Olubuyide *et al*. (1993a,b) carried out a small case–control study in Nigeria to assess the role of HBV and aflatoxins in primary hepatocellular carcinoma. Cases were 22 patients at a university hospital in Ibadan in 1988. Controls were 22 patients from the gastroenterology ward of the same hospital with acid peptic disease unrelated to liver

Table 12. Summary of principal case–control studies on liver cancer and aflatoxins

Reference	Area	Study base	Cases	Controls	Exposure measures	Covariate	Results	Comments
Bulatao-Jayme *et al.* (1982)	Philippines	Three hospitals	PLC; *n* = 90	Patients with normal liver function; *n* = 90 matched by age and sex	Detailed dietary history, linked to measured levels in sample foods. Also biomarkers of aflatoxins	Alcohol and sociodemographic variables	Elevated risks with most aflatoxin-contaminated foods Medium–heavy exp., 15 cases, RR = 14 ($p < 0.05$) Heavy exp., 55 cases, RR = 17 ($p < 0.05$) Urinary aflatoxins B_1 and M_1 were significantly ($p < 0.05$) higher among cases than among controls.	
Lam *et al.* (1982)	Hong Kong	1 university hospital	PLC; *n* = 107 Chinese	Trauma patients, same hospital. *n* = 107 matched by age and sex	Dietary history, linked to earlier market survey of aflatoxin contamination	HBsAg, smoking, alcohol	No differences between cases and controls in reported consumption of different aflatoxin-contaminated foods	
Parkin *et al.* (1991)	Thailand	Three hospitals in Thailand	Cholangiocarcinoma; *n* = 103	Patients visiting clinics or admitted to same hospitals; matched by age, sex and residence to cases; *n* = 103	Dietary history. Aflatoxin–albumin adducts	HBsAg, anti-HBs, anti-HBc, anti-OV, smoking, alcohol	Cases / OR / 95% CI Consumption of presumed aflatoxin-contaminated food: NR / 1.4 / 0.8–2.7 Presence of aflatoxin–albumin adducts: 1 / 1.0 / 0.1–16.0	
Srivatanakul *et al.* (1991b)	Thailand	Three hospitals in Thailand	HCC; *n* = 65	Patients visiting clinics or admitted to same hospitals; matched by age, sex and residence to cases; *n* = 65	Dietary history. Aflatoxin–albumin adducts	HBsAg, anti-HBs, anti-HBc, anti-OV, anti-HCV, smoking, alcohol	Cases / OR / 95% CI Consumption of presumed aflatoxin-contaminated food: NR / 1.9 / Not significant Presence of aflatoxin–albumin adducts: 8 / 1.0 / 0.4–2.7	
Olubuyide *et al.* (1993a)	Nigeria	Hospital in Ibadan	Primary HCC diagnosed in 1988; *n* = 22	Matched patients from gastroenterology ward; *n* = 22	Serum levels of aflatoxin	HBsAg measured but not included in analysis of aflatoxins	High aflatoxin levels were detected in 5 cases and 1 control ($p < 0.05$)	2 of these 5 cases were HBsAg-negative

Table 12 (contd)

Reference	Area	Study base	Cases	Controls	Exposure measures	Covariate	Results				Comments
Mandishona *et al*. (1998)	South Africa	Two hospitals in one province of South Africa	HCC; *n* = 24	Two control series: one hospital-based (trauma or infection patients), *n* = 48; family-based (including related and unrelated family members), *n* = 75	Measured AFB_1–albumin adducts	Several measured, but not used in analysis of aflatoxin	Median levels of AFB_1–albumin adducts were lower among cases than among both sets of controls				High risks of HCC found for HBsAg-positive subjects, alcohol, and iron overload. Questionable comparability of hospital control series, and possible over-matching with family control series
Omer *et al*. (2001)	Sudan	Residents of two regions of Sudan	Cases of HCC diagnosed in 5 out of 6 hospitals in Khartoum; *n* = 150	Community-based, selected from 'sugar shop' registries in same regions as cases; *n* = 205	Detailed diet history for peanut butter, and for storage of peanuts	HBsAg, anti-HCV, smoking alcohol, $GSTM_1$ genotype		Cases	OR	95% CI	Questionable comparability of cases and controls *Test for trend statistically significant **Test for trend among $GSTM_1$-null genotype subjects statistically significant
							Highest quartile of peanut butter intake*	63	3.0	1.6–5.5	
							Humid storage	99	1.6	1.1–2.5	
							Highest quartile of peanut butter intake + $GSTM_1$-null genotype**	NR	17	2.7–105	

AFB_1, aflatoxin B_1; AFM_1, aflatoxin M_1; CI, confidence interval; GST, glutathione *S*-transferase; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; NR, not reported; OR, odds ratio; PLC, primary liver cancer; RR, relative risk

diseases and matched to cases for sex and age. Blood samples were collected after subjects were on hospital diet for one week and were analysed for HBsAg and a number of aflatoxins (B_1, B_2, M_1, M_2, G_1, G_2) and aflatoxicol. HBsAg was detected in 16 cases and 8 controls. Elevated levels of aflatoxins were detected in five (23%) cases and one (5%) control, the difference being significant ($p < 0.05$). [The Working Group questioned the comparability of cases and controls.]

Mandishona *et al*. (1998) carried out a small case–control study in South Africa aiming primarily to determine the role of dietary iron overload in the etiology of HCC. They also collected information on exposure to aflatoxin B_1 and reported risks in relation to this. Cases were 24 consecutive patients with HCC in two hospitals of one province of South Africa. Two control series were assembled. A matched (sex, age, race) series of 48 (two controls per case) was selected from patients hospitalized mainly with trauma or infection. In addition, 75 relatives and family members of the cases constituted a second control series. Interviews were conducted and blood samples taken. Laboratory analyses yielded measures of serum aflatoxin B_1–albumin adducts, iron overload, HBsAg, anti-HCV and other biochemical parameters. The median level of aflatoxin B_1–albumin adducts (pg/mg) was lower among cases (7.3; range 2.4–91.2) than among hospital controls (21.7; range 0–45.6) and family controls (8.7; range 0.7–82.1). Several other parameters (HBsAg, serum ferritin) were higher among cases than controls. [The Working Group noted that the comparability of cases and controls was questionable for the purpose of investigating carcinogenicity of aflatoxin. The measure used to compare aflatoxin levels between groups — the median — may fail to reflect the numbers with high values in the different groups.]

Omer *et al*. (2001) conducted a case–control study in Sudan to assess the association between peanut butter intake as a source of aflatoxins and the *GSTM1* genotype in the etiology of HCC. Cases were 150 patients with HCC who were diagnosed in five out of six hospitals of Khartoum and whose place of residence was in either western Sudan, about 650 km from Khartoum or central Sudan, about 500 km from Khartoum. Controls were 205 residents of the two study areas, and selected by a two-stage process, the second stage of which involved random selection from local village 'sugar shops'. These lists are thought to be comprehensive. Data collection involved a questionnaire which included a particularly detailed history of peanut butter consumption and information on potential confounders. The peanut butter history was transformed into a quantitative cumulative index. Usable blood samples were analysed for HBsAg and anti-HCV (115 cases and 199 controls) and genotyped for *GSTM1* (110 cases and 189 controls). Cases consumed more peanut butter than controls. There was a clear dose–response relationship between average peanut butter consumption and risk for HCC. In the highest quartile of consumption, the OR ranged from 3.0 to 4.0 depending on the covariates included in the model, and all were statistically significant. The pattern of risk differed by region. Peanut butter consumption conferred no increased risk in central Sudan, but a very high risk in western Sudan (OR in the highest quartile, 8.7). Aflatoxin contamination of peanut butter was found to be a much greater problem in western Sudan than

in central Sudan (Omer *et al*., 1998). The authors also noted, however, that residents of the two regions are ethnically different, so effect modification by unmeasured genetic or environmental factors cannot be excluded. While *GSTM1* genotype was not a risk factor for HCC, it was a strong effect modifier. The excess risk due to peanut butter consumption was restricted to subjects with *GSTM1*-null genotype; the OR in the highest quartile of peanut butter exposure among *GSTM1*-null subjects was 17 (95% CI, 2.7–105).

2.4 Limitations of recent studies

While recent studies have incorporated many methodological improvements over studies described in the previous monograph on aflatoxins (IARC, 1993), there nevertheless remain certain problems that limit our ability to fully understand the role of aflatoxins in liver carcinogenesis.

Many recent studies have used HBsAg as the marker of exposure to HBV. However, among liver cancer cases that are negative for HBsAg, HBV DNA can be detected in 33% (serum) and 47% (liver) of the cases, notably those from areas of high viral prevalence. Similarly, HCV RNA can be found in 7% (serum) and 26% (liver) of anti-HCV-negative liver cancer cases (Bréchot *et al.*, 1998). Thus studies relying on HBsAg or anti-HCV measurements may underestimate viral exposure and this may affect an evaluation of interaction between hepatitis viruses and aflatoxin (Paterlini *et al.*, 1994; Kew *et al.*, 1997; Kazemi-Shirazi *et al.*, 2000).

Biomarkers of exposure to aflatoxin have been increasingly used to assess aflatoxin exposure. However, measurable urinary metabolites of aflatoxin or aflatoxin–albumin adducts in serum reflect only exposures in a recent period (days or weeks), and these may not be related to exposures during the etiologically relevant period (years earlier). Moreover, it is unclear whether the presence of liver disease before cancer modifies the levels of the markers found in serum or urine. In the presence of liver disease, comparisons of levels of the marker between liver cancer cases and controls may be biased. Follow-up studies of either general populations in areas of different aflatoxin exposure or of HBsAg carriers investigated with repeated measurements of aflatoxin biomarkers have not yet accumulated follow-up periods that are long enough to minimize the possibility that pre-existing liver disease led to bias in measurements of biomarker levels.

Dietary questionnaires and food measurement surveys at the population level, often used to estimate aflatoxin exposure, provide crude measurements and may fail to account for secular trends in exposure or individual variations in exposure. Similarly, mortality rates used in ecological studies to characterize regions at variable risk of liver cancer may suffer from misclassification of the diagnosis and reporting systems in some countries.

3. Studies of Cancer in Experimental Animals

Studies on the carcinogenicity of aflatoxins in experimental animals completed and reported up to 1993 have been previously evaluated (IARC, 1993). Oral administration of aflatoxin mixtures and aflatoxin B_1 in several strains of rats, hamsters, salmon, trout, ducks, tree shrews and monkeys induced benign and malignant hepatocellular and/or cholangiocellular tumours. Orally administered aflatoxin B_1 did not induce liver tumours in mice. Renal cell tumours were also found in rats following oral administration, while intraperitoneal administration to adult mice increased the incidence of lung adenomas. Intraperitoneal administration of aflatoxin B_1 to pregnant and lactating rats induced benign and malignant liver tumours in mothers and their progeny. Oral administration of aflatoxin B_2 to rats induced liver adenomas, while intraperitoneal administration induced a low incidence of hepatocellular carcinoma. Oral administration of aflatoxin G_1 induced hepatocellular adenomas and carcinomas and renal cell tumours in rats and liver-cell tumours in trout; however, the responses were less than with aflatoxin B_1 at the same dose level. A similar pattern was reported for aflatoxin M_1 and its metabolite aflatoxicol, while aflatoxin Q_1 induced a higher incidence of hepatocellular carcinoma in trout than aflatoxin B_1. Aflatoxin M_1 induced fewer hepatocellular carcinomas following oral administration to rats and trout than aflatoxin B_1 given at an equivalent dose by the same route. Aflatoxicol induced hepatocellular carcinomas in both species; the tumour incidences were lower than that in animals treated with aflatoxin B_1 at the same dose level.

The previous evaluations of aflatoxins (IARC, 1993) were that evidence for carcinogenicity in experimental animals was *sufficient* for aflatoxins B_1, G_1 and M_1, limited for aflatoxin B_2 and *inadequate* for aflatoxin G_2.

This monograph considers only relevant carcinogenicity studies published since 1993.

3.1 Intraperitoneal administration

3.1.1 *Transgenic mouse*

Groups of 11 wild-type $F_1 \times F_1$ (C57BL/6 × CBA) and 12 transgenic mice over-expressing porcine transforming growth factor β_1 (TGF-β_1) [sex unspecified], seven days of age, received aflatoxin B_1 as a single intraperitoneal dose of 6 μg/kg bw. No spontaneous tumours were detected after 12 months in either nine control wild-type or 19 control transgenic mice. Adenomas [assumed to be hepatocellular adenomas] (2/12 and 3/11) and hepatocellular carcinomas (1/12 and 0/11) were detected after 12 months in the liver of the aflatoxin-treated transgenic and wild-type mice, respectively (Schnur *et al*., 1999). [The Working Group noted the limited reporting of this study.]

3.2 Oral administration of ammoniated forms

3.2.1 *Rat*

In a study to investigate the effect of ammoniation on the carcinogenicity of aflatoxin B_1-contaminated cakes, male and female Wistar WAG and Fischer 344 rats, 4–5 weeks of age, were fed: diet containing 30% peanut oil cake contaminated with 1000 ppb [µg/kg] aflatoxin B_1 and 170 ppb aflatoxin G_1; contaminated diet treated with pressurised ammonia gas (2 bar; 200 kPa) for 15 min at 95 °C (140 ppb aflatoxin B_1 and 20 ppb aflatoxin G_1); or contaminated diet treated with ammonia at a pressure of 3 bar (300 kPa) for 15 min at 95 °C (60 ppb aflatoxin B_1 and 10 ppb aflatoxin G_1). A control group received a diet containing 30% of uncontaminated peanut oil cakes (later determined to contain ~50 ppb aflatoxin B_1); this was reduced to 25% of diet after one month due to greater consumption than the other diets. Deaths occurred among rats receiving the contaminated, untreated diet (10% male Fischer 344, 25% female Fischer 344, 15% male Wistar and 25% female Wistar). Incidences of hepatic tumours at the 12-month termination are given in Table 13. Treatment with ammonia at 2 bar (200 kPa) greatly reduced the carcinogenic potential of the contaminated diet. Treatment with ammonia at 3 bar (300 kPa) eliminated induction of tumours occurring at 12 months (Frayssinet & Lafarge-Frayssinet, 1990).

Table 13. Effect of ammonia treatment on incidence of hepatic tumours in rats fed aflatoxin-contaminated diets for 12 months

Strain	Sex	Contaminated diet	Contaminated/ treated diet (200 kPa)	Contaminated/ treated diet (300 kPa)	Control
Fischer 344	Males	18/19	0/20	0/15	0/20
	Females	5/11	0/10	0/19	0/10
Wistar WAG	Males	17/17	2/20	0/31	0/20
	Females	9/11	1/10	0/20	0/10

From Frayssinet & Lafarge-Frayssinet (1990)

3.2.2 *Trout*

The effect of ammoniation on the hepatocarcinogenic potential of aflatoxin B_1 was investigated in Mount Shasta strain rainbow trout. Duplicate groups of 80 trout (average weight 63 g) were randomly distributed into ~380-L tanks and fed uncontaminated corn or corn contaminated with 180 µg/kg aflatoxins (B_1, 160 µg/kg; B_2, 10 µg/kg; G_1, 9 µg/kg; G_2, trace) treated by ammoniation or untreated, in the following diets for 12 months: uncontaminated untreated corn; uncontaminated treated corn; contaminated untreated corn; and contaminated treated corn. This corn was mixed with modified basal

diet and controls received only the basal diet (Table 14). Ten fish were removed from each tank for histopathological evaluation at four and eight months and the remaining fish were held for 12 months. Liver nodules of doubtful classification and five randomly sampled livers at each sampling date were examined histopathologically. At eight months, fish fed contaminated untreated corn had a high incidence (19/20) of hepatoma (Brekke *et al.*, 1977). Ammoniation significantly reduced the carcinogenic potential at 12 months of aflatoxin B_1-contaminated corn (Table 14).

Table 14. Incidence of hepatoma in trout fed various diets for 12 months

Diet	Incidence of hepatoma
Basal diet	1/116
Uncontaminated corn untreated	2/115
Uncontaminated ammonia-treated corn	0/111
Contaminated corn untreated	109/112
Contaminated ammonia-treated corn	3/116

From Brekke *et al*. (1977)

In a more recent experiment, trout were given non-fat dried milk power prepared from the milk of cows that had received aflatoxin-contaminated diets. Thirty lactating cows (Holstein-Friesian) were fed a ration containing ammonia-treated (atmospheric pressure at ambient temperature; AP/AT) aflatoxin-contaminated whole cottonseed (aflatoxin B_1, 5200 μg/kg) for seven days followed by an untreated aflatoxin-containing seed for seven days. The final aflatoxin B_1 concentration in the cow ration was 780 μg/kg. In a second experiment, three lactating cows were fed for 10 days a ration containing ammonia-treated (high pressure at high temperature; HP/HT) aflatoxin-contaminated cottonseed (aflatoxin B_1, 1200 μg/kg), incorporated at 25% (w/w) of the total dry weight of the ration. Then, they were fed for 10 days a ration containing untreated aflatoxin-contaminated cottonseed (aflatoxin B_1, 1200 μg/kg), incorporated at 25% (aflatoxin B_1, 300 μg/kg, in final ration). Milk was collected daily from days 3 to 7 (for each period) when cows were fed the AP/AT material and from days 3 to 10 (for each period) when they were fed the HP/HT material. The milk was processed to prepare non-fat dried milk powder, which was fed as 25% of the diet to rainbow trout (*Oncorhynchus mykiss*) for 12 months. The aflatoxin M_1 levels in the milk powders from cows given untreated and treated seed were: AP/AT, 85 and < 0.05 μg/kg; and HP/HT, 32 and < 0.05 μg/kg, respectively. AP/AT treatment reduced the liver tumour incidence to 2.5% compared with 42% in the trout fed the milk from the cows that received the untreated cottonseed. In positive controls, feeding aflatoxin B_1 (4 μg/kg) continuously for 12 months resulted in a 34% tumour incidence, while feeding for two weeks a diet containing 20 μg/kg aflatoxin B_1 resulted in an incidence of 37% of liver tumours, and feeding of 80 μg/kg or 800 μg/kg

aflatoxin M_1 resulted in tumour incidences of 5.7 and 50%, respectively, after nine months. In the separate HP/HT experiment, no tumours were observed in the livers of the trout fed diets containing milk from either the ammonia-treated or untreated source or the control diet containing 8 µg/kg aflatoxin M_1. In positive controls fed 64 µg/kg aflatoxin B_1 for two weeks, tumour incidence was 29% after 12 months. It was concluded that neither aflatoxin M_1 at 8 µg/kg nor any HP/HT-derived aflatoxin derivatives carried over into milk represented a detectable carcinogenic hazard to trout (Bailey *et al*., 1994a).

3.3 Carcinogenicity of metabolites

Trout: Fry (*O. mykiss*, Shasta strain) were fed Oregon Test Diet (OTD) containing 0, 4, 8, 16, 32 or 64 ng aflatoxin B_1 or aflatoxicol per g dry weight of diet for two weeks. Each dietary group consisted of 400 treated fish or 200 control fish. The experiment was terminated after nine months. The incidence of hepatic tumours in the groups given the 4, 8, 16, 32 and 64 ng/g diet was 25/382 (7%), 98/387 (25%), 194/389 (50%), 287/389 (74%), 302/383 (80%) for aflatoxin B_1 and 57/200 (29%), 143/345 (41%), 183/386 (47%), 255/383 (66%) and 291/390 (75%) for aflatoxicol. No hepatic tumours (0/192) were seen in the controls. In the second protocol, quadruplicate groups of 120 eggs (21-day-old embryos) were exposed to various concentrations (0.01, 0.025, 0.05, 0.1, 0.25, 0.5 µg/mL) of aflatoxin B_1 or aflatoxicol for 1 h. At swimup (after hatching and yolk sac absorption), 360 healthy fry per treatment group (320 and 240 fish from the 0.5 ppm aflatoxin B_1- and aflatoxicol-treated embryos, respectively) were fed the OTD diet for 13 months. There was a dose-related incidence of hepatic tumours: 15/346 (4%), 59/348 (17%), 131/343 (38%), 191/343 (57%), 254/347 (73%), and 252/313 (80%) for the six aflatoxin concentrations and 28/347 (8%), 157/346 (45%), 245/353 (69%), 276/355 (78%), 275/338 (81%) and 148/220 (67%) for the six aflatoxicol concentrations. Aflatoxicol induced a slightly higher hepatic tumour response in fry and fish embryos than did aflatoxin B_1 (Bailey *et al*., 1994b).

Groups of 120 rainbow trout fry, weighing 1.2 g, were treated with concentrations of aflatoxin B_1 and aflatoxicol ranging from 4 to 64 ng/g of diet and of aflatoxin M_1 and aflatoxicol M_1 ranging from 80 to 1280 ng/g of diet for two weeks. All the fry were then maintained on the control diet until termination at one year. The tumour responses relative to aflatoxin B_1 were: aflatoxin B_1, 1.00; aflatoxicol, 0.936; aflatoxin M_1, 0.086; and aflatoxicol M_1, 0.041. The authors also monitored DNA-adduct formation and concluded that the differences in tumour response were largely accounted for by differences in uptake and metabolism leading to DNA adduct formation, rather than differences in tumour-initiating potency per DNA adduct (Bailey *et al*., 1998).

3.4 Administration with known carcinogens and other modifying factors

3.4.1 *Viruses*

Transgenic mouse: Hepatitis B virus-positive (HBV+) C57BL/6 mice were bred with *TP53*-null mice (*TP53*$^{-/-}$) to produce *TP53*$^{+/-}$, HBV+ mice. These mice and control litter mates (*TP53*$^{+/+}$, HBV+ and *TP53*$^{+/-}$, HBV–) were randomly divided into groups of 16–24 animals. Approximately half of the animals in each group were females. The experimental group received a single intraperitoneal injection of 10 mg/kg bw aflatoxin B_1 in tricaprylin, while the controls received tricaprylin alone at the age of one week. Surviving animals were sacrificed at 13 months and assessed for HBV positivity by HBsAg expression. The incidence of hepatocellular tumours of Beckers classification grade 2 or higher [adenomas and carcinomas] was 100% in males that were heterozygous for the *TP53* allele and that carried HBV and received aflatoxin B_1 and was 62.5% in males that were homozygous for the wild-type *TP53* allele and had both risk factors (HBV and aflatoxin B_1). The presence of HBV without aflatoxin in heterozygous animals was more potent (25%) than the presence of aflatoxin without HBV (14.2%). The wild-type *TP53* was capable of suppressing tumours when animals were exposed to either risk factor alone (0%). In a companion set of experiments reported later, the relative effect of a mutant allele for *TP53* at serine 246 (the mouse homologue to the human *TP53* 249^{ser} mutation) on the risk factors described above was examined. The 246^{ser} mutation, when present in heterozygous *TP53* male animals, led to the development of tumours in 25% of the animals even without virus or aflatoxin. In animals heterozygous for *TP53* with the 246^{ser} mutation, 71% of the animals receiving aflatoxin B_1 and not carrying HBV had tumours, whereas in the previous study, the heterozygous wild-type allele was associated with 14.2% tumours. Female mice in both studies had fewer tumours but exhibited similar patterns of response. The presence of the *TP53* 246^{ser} mutant not only enhanced the synergistic effect of HBsAg and aflatoxin B_1 but also increased tumorigenesis in aflatoxin B_1-treated mice not expressing HBsAg (Ghebranious & Sell, 1998a,b).

*Tree shrew (*Tupaia belangeri chinensis*)*: Male and female tree shrews, weighing 100–160 g [age unspecified], were divided into four groups. Normal and HBV (human)-infected animals were fed aflatoxin B_1 at 150 μg/kg bw per day in milk on six days per week for 105 weeks. Animals were held until 160 weeks. Hepatocellular carcinomas developed in 67% (14/21) of both male (5/10) and female (9/11) combined HBV-infected/aflatoxin B_1-treated animals and in 30% (3/10) animals treated with aflatoxin B_1 alone (male, 1/6; female, 2/4). The average time for development of hepatocellular carcinoma for males and females combined was significantly reduced ($p < 0.01$) with combined HBV-infection and aflatoxin B_1 treatment (120.3 ± 16.6 weeks) compared with aflatoxin B_1 treatment alone (153 ± 5.8 weeks) (Li *et al*., 1999).

*Woodchuck (*Marmota monax*)*: Woodchucks were infected with the woodchuck hepatitis virus (WHV; closely related to HBV) at 2–7 days of age. From the age of 12 months, groups of six male and six female WHV-positive and six male and six female WHV-negative animals were fed 50 μg/kg bw aflatoxin B_1 (< 99% pure) in bananas, five

days per week for four months followed by treatment with 20 µg/kg bw aflatoxin B_1, five days per week for life. Two untreated groups (WHV-positive and -negative) served as the controls. Woodchucks infected with WHV with or without aflatoxin B_1 treatment developed preneoplastic foci, hepatocellular adenomas and carcinomas between 6 and 26 months after commencing the treatment. Liver tumours were observed by ultrasound at 25 months in 5/9 animals infected with WHV and at 11 months in 1/11, at 19 months in 4/10 and at 25 months in 2/5 animals that received the combined WHV/aflatoxin treatment. No liver tumours were diagnosed in aflatoxin B_1-treated or untreated control animals. The combined treatment resulted in earlier tumour appearance than with WHV alone (Bannasch *et al*., 1995).

4. Other Data Relevant to an Evaluation of Carcinogenicity and its Mechanisms

4.1 Absorption, distribution, metabolism and excretion

4.1.1 *Humans*

Rigorous quantitative comparisons of dietary intakes and aflatoxin metabolites in body fluids following absorption and distribution are lacking. As noted in the previous monograph (IARC, 1993), aflatoxin M_1 concentrations in urine and human milk have been correlated with dietary aflatoxin intake. However, studies of human exposure have yielded quantitatively very different correlations between aflatoxin concentrations in foods and either aflatoxin–protein or aflatoxin–DNA adducts in urine and sera (Hall & Wild, 1994). Hudson *et al*. (1992) very carefully measured aflatoxin intake based on plate foods in a village in The Gambia. They found intakes less than those estimated from aflatoxin–serum and urinary adduct levels in the same individuals. In humans, as with other species, the DNA binding and carcinogenicity of aflatoxin B_1 result from its conversion to the 8,9-epoxide by cytochrome P450 (CYP) enzymes (Essigman *et al.*, 1982). There is individual variability in the rate of activation of aflatoxin, including between children and adults, which may be material to the pharmacokinetics (Ramsdell & Eaton, 1990; Wild *et al.*, 1990). The pharmacokinetics of aflatoxins in humans are still not clearly known.

Factors that explain variation in response to aflatoxin between individual humans, animal species and strains include the proportion of aflatoxin metabolized to the 8,9-epoxide (mainly by CYP enzymes) relative to the other much less toxic metabolites and the prevalence of pathways forming non-toxic conjugates with reduced mutagenicity and cytotoxicity. Several excellent reviews have been published on the metabolism of aflatoxins since the last IARC monograph on this topic (Eaton & Gallagher 1994; McLean & Dutton 1995; Guengerich *et al*., 1998).

The 8,9-epoxide of aflatoxin B_1 is short-lived but highly reactive and is the main mediator of cellular injury (McLean & Dutton, 1995). Formation of DNA adducts of aflatoxin B_1-epoxide is well characterized. The primary site of adduct formation is the *N*7 position of the guanine base (Guengerich *et al.*, 1998).

The metabolism of aflatoxins in humans has been extensively studied and the major CYP enzymes involved have been identified as CYP1A2 and CYP3A4 (Gallagher *et al*., 1996; Ueng *et al*., 1998). CYP3A4 mediates formation of the *exo*-epoxide and aflatoxin Q_1 while CYP1A2 can generate some *exo*-epoxide but also a high proportion of *endo*-epoxide and aflatoxin M_1. In-vitro evidence that both CYP3A4 and 1A2 are responsible for aflatoxin metabolism in humans has been substantiated by biomarker studies. Aflatoxins M_1 and Q_1, produced by CYP1A2 and 3A4, respectively, are present in the urine of individuals exposed to aflatoxin (Ross *et al*., 1992; Qian *et al*., 1994). The *N*7-guanine adducts of aflatoxin are generated primarily by the *exo*-8,9-epoxide, from which yields of adduct are > 98% (Guengerich *et al.*, 1998). The overall contribution of the above enzymes to aflatoxin B_1 metabolism *in vivo* will depend not only on their affinity but also on their expression in human liver, where CYP3A4 is predominant. CYP3A5, in contrast to CYP3A4, metabolizes aflatoxin B_1 mainly to the *exo*-8,9 epoxide but is about 100-fold less efficient in catalysing 3-hydroxylation of aflatoxin B_1 to yield the aflatoxin Q_1 metabolite (Wang *et al*., 1998). Hepatic CYP3A5 expression differs markedly between individuals, with a proportion of the population, dependent on ethnic group, showing no expression; in particular, 40% of African Americans do not express this enzyme. Therefore, differences in expression of CYP3A5 could influence susceptibility to aflatoxins. Recently, polymorphisms have been identified in the promoter region of CYP3A5 leading to alternative splicing and truncated protein (Hustert *et al*., 2001; Kuehl *et al*., 2001). The role of these polymorphisms in susceptibility to aflatoxins is currently unknown.

CYP3A7 (also called P450 HFLa) is a major form of cytochrome P450 in human fetal liver, which has the capacity to activate aflatoxin B_1 to the 8,9-epoxide (Kitada *et al*., 1989, 1990). This is consistent with the detection of aflatoxin–albumin adducts in the cord blood of newborns whose mothers were exposed to dietary aflatoxin in The Gambia (Wild *et al*., 1991). Recombinant CYP3A7 conferred sensitivity to aflatoxin B_1 in transfected Chinese hamster lung cells (Kamataki *et al*., 1995).

In humans, the reactive *exo*- and *endo*-epoxides of aflatoxin B_1 can be detoxified via a number of pathways. One route is glutathione *S*-transferase (GST)-mediated conjugation to reduced glutathione (GSH) to form aflatoxin B_1 *exo*- and *endo*-epoxide–GSH conjugates (Guengerich *et al.*, 1998). The *exo*- and *endo*-epoxides can also hydrolyse rapidly by a non-enzymatic process to an 8,9-dihydrodiol that in turn undergoes slow, base-catalysed ring opening to a dialdehyde phenolate ion (Johnson *et al*., 1996; Johnson & Guengerich, 1997). The dialdehydes from aflatoxins B_1 and G_1 form Schiff bases with primary amine groups such as those in lysine, to yield protein adducts, for example with albumin (Sabbioni *et al*., 1987; Sabbioni & Wild 1991). A further metabolic step involves aflatoxin B_1 aldehyde reductase (AFB_1-AR) which catalyses the NADPH-dependent

reduction of the dialdehydic phenolate ion to a dialcohol; this enzyme has been characterized in both rats and humans (Hayes *et al*., 1993; Ireland *et al*., 1998; Knight *et al*., 1999).

The role of epoxide hydrolase in hydrolysis of aflatoxin B_1 8,9-epoxide has been investigated (Guengerich *et al.*, 1996; Johnson *et al*., 1997a,b) with respect to the observed association between epoxide hydrolase genotype and risk for hepatocellular carcinoma in aflatoxin-exposed populations (McGlynn *et al*., 1995; Tiemersma *et al*., 2001). If the enzyme is involved, its contribution may be limited, given the rapid non-enzymatic hydrolysis mentioned above (Guengerich *et al*., 1998).

Oltipraz, an antischistosomal drug, acts as a potent inhibitor of aflatoxin-induced hepatocarcinogenesis in animal models. A total of 234 healthy adults from Qidong (China) were assigned to receive 125 mg oltipraz daily, 500 mg oltipraz weekly or a placebo. Urinary aflatoxin metabolites were quantified by sequential immunoaffinity chromatography and liquid chromatography coupled to mass spectrometry or fluorescence detection. One month of weekly administration of 500 mg oltipraz led to a 51% decrease in the amount of aflatoxin M_1 excreted in urine compared with administration of a placebo ($p = 0.030$), but it had no effect on concentrations of aflatoxin–mercapturic acid ($p = 0.871$). Daily intervention with 125 mg oltipraz led to a 2.6-fold increase in median aflatoxin–mercapturic acid excretion ($p = 0.017$) but had no effect on excreted aflatoxin M_1 levels ($p = 0.682$). It was concluded that the higher dose of oltipraz inhibited aflatoxin activation, whereas the lower dose increased GSH conjugation of aflatoxin 8,9-epoxide (Wang *et al*., 1999a). Among other things, this clinical trial demonstrates that the results of studies conducted *in vitro* on the major pathways of aflatoxin processing (discussed below) are consistent with human data.

Kirby *et al*. (1993) examined liver tissues from 20 liver cancer patients from Thailand, an area where exposure to aflatoxin occurs. The activity of hepatic CYP isoenzymes and GST was examined and compared with the in-vitro metabolism of aflatoxin B_1. There was considerable inter-individual variation in activity of CYP enzymes, including CYP3A4 (57-fold), CYP2B6 (56-fold) and CYP2A6 (120-fold). In microsomal preparations from liver tumours, metabolism of aflatoxin B_1 to the 8,9-epoxide and aflatoxin Q_1 (the major metabolites) was related to the concentration of CYP3A3/4 and CYP2B6. There was significantly reduced activity of major CYP proteins in microsome preparations from liver tumours compared with those from adjacent non-tumour areas in the liver. The major classes of cytosolic GSTs (α, μ and π) were also analysed in normal and tumorous liver tissue. The activity of α and μ class proteins was decreased and π increased in the majority of tumour cytosols compared with normal liver. Cytosolic GST activity was significantly lower in liver tumours than in normal liver. There was no detectable conjugation of aflatoxin B_1 8,9-epoxide to GSH by microsomal preparations from either normal liver or liver tumour tissue.

Heinonen *et al.* (1996) studied aflatoxin B_1 metabolism in human liver slices from three donors by incubating the tissue with 0.5 μM [^{3}H]aflatoxin B_1 for 2 h. The rates of oxidative metabolism of aflatoxin B_1 to aflatoxins Q_1, P_1 and M_1 were similar to those

observed in rat liver slices, albeit with significant interindividual variation. GSH-conjugate formation was not detected in the human liver samples.

It is probable that the apparent discrepancies between studies showing the elimination of mercapturic acids in the urine of aflatoxin-exposed individuals (Wang *et al*., 1999a) and the apparent lack of formation of glutathione conjugates in cytosolic incubations with aflatoxin B_1 8,9-epoxide (Heinonen *et al.*, 1996) are due to differences in sensitivity of the analytical methods employed.

Rodent studies (see below) have demonstrated that viral damage to the liver affects the metabolism of aflatoxin. Kirby *et al*. (1996a) examined the expression of CYP enzymes in sections of normal human liver and in livers with hepatitis and cirrhosis. By use of immunohistochemical techniques, it was shown that in sections infected with hepatitis B virus (HBV) or hepatitis C virus (HCV), the concentration of CYP2A6 was increased in hepatocytes immediately adjacent to areas of fibrosis and inflammation. In the same tissues, CYP3A4 and CYP2B1 were somewhat increased and CYP1A2 was unaffected compared with normal liver. In HCV-infected liver, CYP2A6, CYP3A4 and CYP2B1 were increased in hepatocytes that had accumulated haemosiderin pigment.

4.1.2 *Experimental systems*

(*a*) *Human tissues*

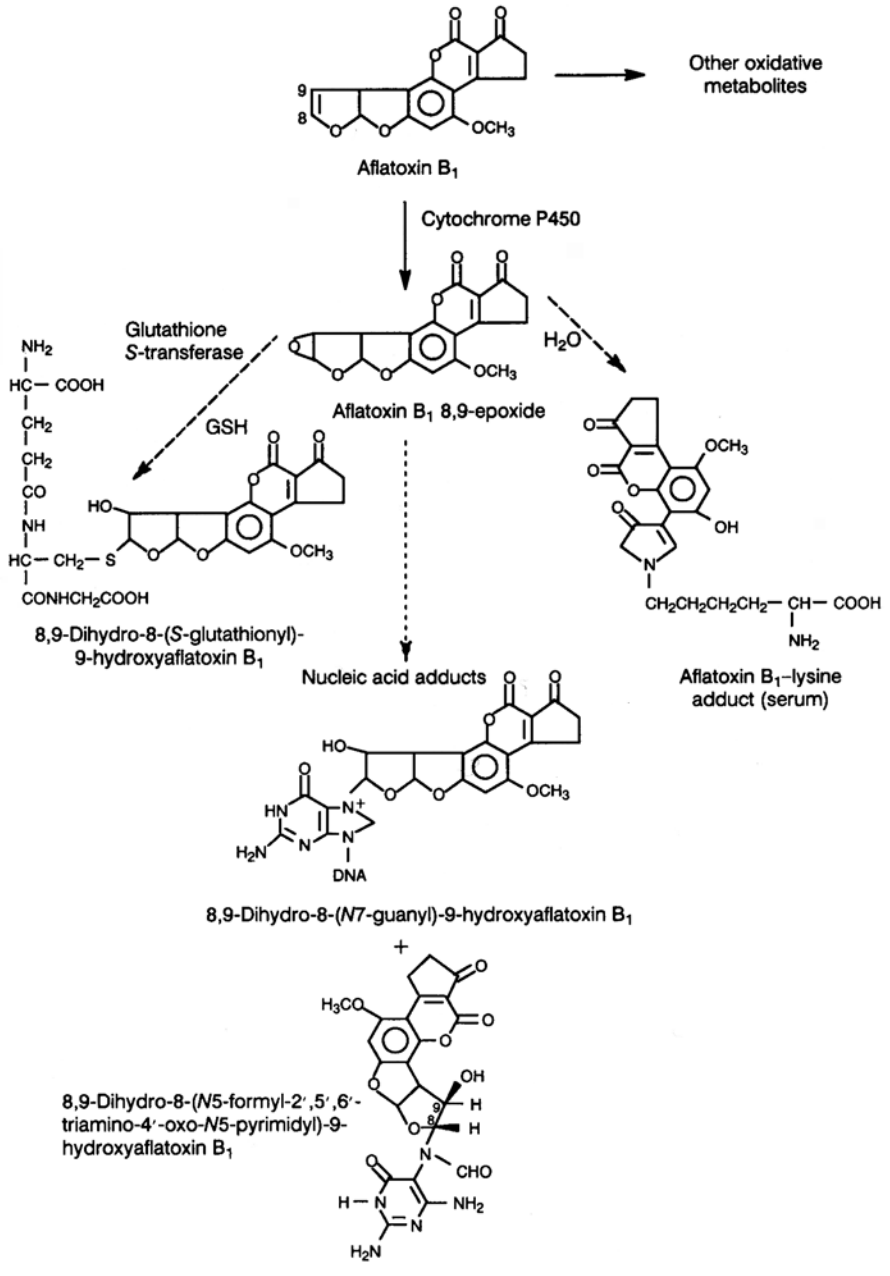
Data published before 1993 were reviewed in *IARC Monographs* Volume 56 (IARC, 1993). The metabolism and major metabolites of aflatoxin B_1 are shown in Figures 3 and 4.

Gallagher *et al.* (1996) studied the kinetics of aflatoxin oxidation in human liver microsomes and in lymphoblastoid microsomes expressing human CYP3A4 and CYP1A2 cDNA: the K_m was 41 μM for CYP1A2 and 140–180 μM (average affinity for two binding sites) for CYP3A4. In the case of CYP3A4, the rate of product formation dropped as the substrate concentration was reduced. In contrast, CYP1A2 has a higher affinity for aflatoxin. In humans, at plausible serum aflatoxin concentrations, the rate of formation of aflatoxin 8,9-epoxide will be determined by both the lower K_m of CYP1A2 and the greater abundance of CYP3A4 in human liver.

The ability of the human lung to metabolize aflatoxin has been studied in the context of the risk of pulmonary carcinogenesis from handling crops contaminated by aflatoxins. Bioactivation of tritiated aflatoxin B_1 was demonstrated in fresh lung preparations from patients undergoing lobectomy for lung cancer. Lipoxygenase and prostaglandin H synthase activity was shown to be primarily responsible for aflatoxin activation, rather than the CYP enzymes, which display a low level of activity in this tissue (Donnelly *et al*., 1996).

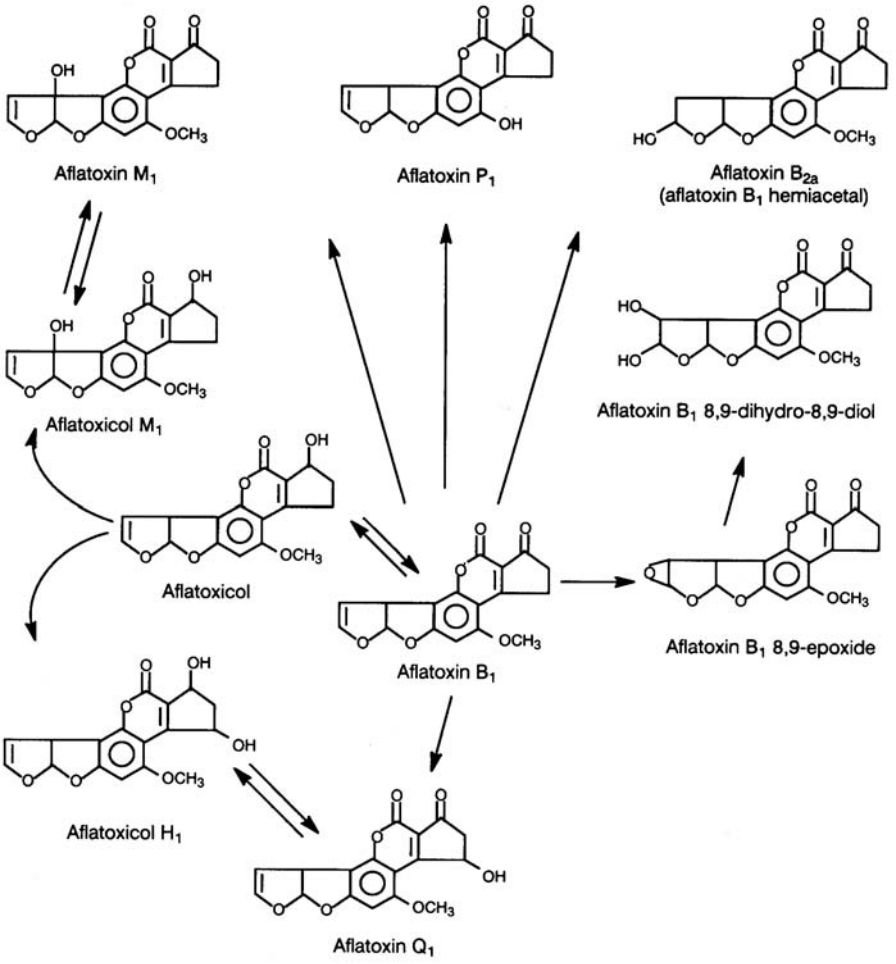
Neal *et al.* (1998) compared the metabolism of aflatoxins B_1 and M_1 *in vitro* using human liver microsomes. Indirect evidence was obtained for metabolism of aflatoxin M_1 to the 8,9-epoxide by trapping the reactive metabolite with Tris and GSH in the presence of mouse cytosolic fraction. Human liver cytosol did not appear to mediate GSH

Figure 3. Metabolic activation of aflatoxin B_1 to the 8,9-epoxide, leading to binding to glutathione, DNA and serum albumin



From Essigman *et al.* (1982)

Figure 4. Major metabolites of aflatoxin B_1



From Essigman *et al.* (1982)

conjugation of either microsomally activated aflatoxin B_1 or aflatoxin M_1. An interesting observation was the cytotoxicity of low doses of aflatoxin M_1 (≥ 0.5 μg/mL) to lymphoblastoid cells in the absence of metabolic activation, which was not observed with aflatoxin B_1.

A non-tumorigenic SV40-immortalized human liver epithelial cell line expressing human CYP1A2 cDNA mediated the formation of both aflatoxin B_1– and aflatoxin M_1–DNA adducts, suggesting that aflatoxin B_1 hydroxylation to aflatoxin M_1 can subsequently lead to DNA damage (Macé *et al.*, 1997).

As mentioned above, human cytosolic fractions or liver slices *in vitro* show little detectable conjugation of aflatoxin B_1 8,9-epoxide. Purified recombinant human α-class

GSTs, namely hGSTA1-1 and hGSTA2-2, also lack significant activity (Raney *et al*., 1992; Buetler *et al*., 1996; Johnson *et al*., 1997a,b). Some conjugating activity was expressed by human μ-class GSTs M1a-1a and M2-2, although mostly towards the *endo*-epoxide (Raney *et al*., 1992).

Langouët *et al*. (1995) investigated metabolism of aflatoxin B_1 in primary human hepatocytes from eight human liver donors with or without pretreatment by oltipraz. Parenchymal cells obtained from the three GSTM1-positive livers metabolized aflatoxin B_1 to aflatoxin M_1 and to aflatoxin B_1–glutathione conjugates, but no such conjugates were formed in the cells lacking GSTM1. Although oltipraz treatment of the cells induced GSTs A2, A1 and M1, it resulted in decreased formation of aflatoxin M_1 and aflatoxin B_1 oxides due to inhibition of CYP1A2 and CYP3A4.

(*b*) *Experiments on animals and animal tissues*

Kirby *et al*. (1996b) demonstrated that viral infections causing liver injury alter the activity of aflatoxin-metabolizing enzymes in human liver. This has also been shown in transgenic mice that overproduce the HBV large envelope protein, which results in progressive liver cell injury, inflammation and regenerative hyperplasia. The activity of CYP2A5 and CYP3A and a GSTα isoenzyme was examined in these mice. Increased activity and altered distribution of CYP2A5 were shown to be associated with the development of liver injury. The amount of CYP3A was also increased, while GSTα enzyme concentrations were the same in transgenic mice and in otherwise isogenic, non-transgenic mice (Kirby *et al*., 1994a).

Fetal rat liver contains a GST that forms a conjugate with aflatoxin B_1 8,9-epoxide, identified as a GSTα. By means of immunoblotting and enzyme assays it was shown that liver from adult female rats contains concentrations of one of the enzyme subunits (Yc_2) about 10-fold higher than those in liver from adult male rats. This may contribute to the relative insensitivity of female rats to aflatoxin B_1 (Hayes *et al*., 1994).

In addition to CYP-mediated activation, aflatoxin B_1 8,9-epoxide can also be formed through metabolism by lipoxygenase and prostaglandin H synthase isolated from guinea-pig tissues and ram seminal vesicles, respectively (Battista & Marnett, 1985; Liu & Massey, 1992). In some organs, for example guinea-pig kidney, the contributions of CYP and prostaglandin H synthase to formation of the epoxide are similar (Liu *et al*., 1990).

There are marked species differences in sensitivity to aflatoxin carcinogenesis (Gorelick, 1990; Eaton & Gallagher, 1994; Eaton & Groopman, 1994). For example, the adult mouse is almost completely refractory to tumour formation except under conditions of partial hepatectomy or liver injury through expression of HBV antigens. In contrast, the rat is extremely sensitive (see Section 3). A considerable part of this interspecies variation is now understood in terms of differences in activity of aflatoxin-metabolizing enzymes in the pathways described above. Microsomal preparations from mice actually exhibit higher specific activity for aflatoxin B_1 8,9-epoxide production than the rat (Ramsdell & Eaton, 1990). However, in the mouse, the resistance to aflatoxin carcinogenesis is largely if not exclusively explained by the constitutive hepatic expression of

an α-class GST, mGSTA3-3, which has high affinity for aflatoxin B_1 8,9-epoxide (Buetler & Eaton, 1992; Hayes *et al*., 1992). In contrast, rats do not constitutively express a GST isoform with high epoxide-conjugating activity but do express an inducible α-class GST (rGSTA5-5) with high activity. The induction of this enzyme plays a major role in the resistance of rats to aflatoxin B_1-induced hepatocarcinogenicity following treatment with enzyme inducers including oltipraz, ethoxyquin and butylated hydroxyanisole (Kensler *et al*., 1986, 1987; Hayes *et al*., 1991, 1994).

A cross-species study of rats (Fischer 344, Sprague-Dawley and Wistar), mice (C57BL), hamsters (Syrian golden) and guinea-pigs (Hartley) was conducted using doses of aflatoxin B_1 between 1 and 80 μg/kg bw per day for up to 14 days by gavage (Wild *et al*., 1996). Aflatoxin–albumin adducts were measured at 1, 3, 7 and 14 days and hepatic aflatoxin B_1–DNA adducts were measured at the final time point. Both albumin and DNA adducts were formed in the order rat > guinea-pig > hamster > mouse, with similar ratios between the two biomarkers across species, suggesting that the albumin adducts reflected hepatic DNA damage. Calculations from human environmental exposure data and albumin adducts suggested that humans and rats — a sensitive species — have similar formation of albumin adducts for a given exposure to aflatoxin.

In rats, the μ-class enzymes rGSTM2-2 and rGSTM2-3 can conjugate both the *exo*- and *endo*-epoxide of aflatoxin but the latter is the preferred substrate (Raney *et al*., 1992; Johnson *et al*., 1997a). Wang *et al*. (2000) showed that the GST-conjugating ability of the non-human primate, *Macaca fascicularis* (mf), towards the 8,9-epoxide was partially due to a μ-class GST, mfaGSTM2-2, with 96% amino acid homology to the human hGSTM2. The enzyme mfaGSTM2-2 was predominantly active towards the *endo*-epoxide, whereas another enzyme, GSHA-GST, for which the encoding cDNA was not cloned, had activity towards the *exo*-epoxide of aflatoxin. However, the activity was about two orders of magnitude lower than that of the rodent α-class GSTs, mGSTA3-3 and rGSTA5-5.

In direct comparison, human and marmoset (*Callithrix jacchus*) hepatic microsomes had similar rates of oxidation of aflatoxin B_1 to the 8,9-epoxide to those of macaques (*Macaca nemestrina*), but GST activity towards the epoxide was below the detection limit in the former two species (Bammler *et al*., 2000).

Stresser *et al*. (1994a) examined the influence of dietary indole-3-carbinol (found in cruciferous vegetables) on the relative levels of different CYP isozymes known to metabolize aflatoxin B_1 in male Fischer 344 rats. Diets containing 0.2% (w/w) indole-3-carbinol given for seven days were shown to increase the microsomal concentrations of CYP1A1, 1A2 and 3A1/2 (24-, 3.1- and 3.8-fold, respectively, compared with rats receiving a control diet), with a smaller effect on 2B1/2 (1.7-fold) and no effect on CYP2C11. The influence of dietary indole-3-carbinol on the aflatoxin B_1 glutathione detoxication pathway and aflatoxin B_1–DNA adduct formation was also investigated. After seven days of feeding a diet containing indole-3-carbinol (0.2% w/v), rats were administered [^{3}H]aflatoxin B_1 (0.5 mg/kg bw) by intraperitoneal injection and killed after 2 h. The diet with indole-3-carbinol inhibited the formation of aflatoxin B_1–DNA adducts in the liver by 68%, based on analysis of DNA-bound radioactivity (Stresser *et al*., 1994b).

4.2 Toxic effects

4.2.1 *Humans*

Reports of toxic effects of aflatoxins in humans were reviewed in the previous IARC monograph (IARC, 1993).

There are data suggesting that children are more vulnerable than adults to acute hepatotoxicity resulting from ingestion of aflatoxin. In 1988, 13 Chinese children died of acute hepatic encephalopathy in Perak, Malaysia (Lye *et al*., 1995). Common symptoms included vomiting, haematemesis and seizures; jaundice was detected in seven cases and all children had liver dysfuntion with elevated serum concentrations of hepatic enzymes (aspartate aminotransferase and alanine aminotransferase). The deaths occurred 1–7 days after hospital admission and were associated with consumption of Chinese rice noodles shortly before the outbreak. Aflatoxins were found in blood and organs from the children (Chao *et al*., 1991). Pesticides, carbon tetrachloride and mushroom poisons were not found. The flour used to make the noodles was found to contain aflatoxin. Adults who presumably consumed the same contaminated food were not reported to have been affected (Lye *et al*., 1995).

Children suffering from protein-energy malnutrition in developing countries may also be exposed to aflatoxin. In a study conducted in South Africa, aflatoxin concentrations in serum were higher in 74 children with protein-energy malnutrition than in 35 age-matched control children. The control group, however, had a higher concentration of aflatoxins in urine (Ramjee *et al.*, 1992). [Possible explanations for this result are that aflatoxin metabolism is affected in children with protein-energy malnutrition or that malnourished children are more highly exposed.] A second study compared children with protein-energy malnutrition with high (n = 21) and undetectable (n = 15) aflatoxin concentrations in serum and urine. The aflatoxin-positive group of children with protein-energy malnutrition showed a significantly lower haemoglobin level (p = 0.02), longer duration of oedema (p = 0.05), an increased number of infections (p = 0.03) and a longer duration of hospital stay (p = 0.008) than the aflatoxin-negative group (Adhikari *et al*., 1994). This finding confirmed results of an earlier study which suggested that malarial infections were increased in children exposed to aflatoxin, as determined on the basis of the amounts of aflatoxin–albumin adducts (Allen *et al*., 1992). However, a similar study from the Philippines gave inconclusive results (Denning *et al*., 1995). [The Working Group noted that in these studies estimates of aflatoxin exposures were not available and that possible confounders were not considered.]

4.2.2 *Experimental systems*

No primary studies on the toxicity of aflatoxins were found other than those summarized in IARC (1993) and Eaton and Groopman (1994).

Experimental carcinogenicity studies with aflatoxin B_1 reported previously (IARC, 1993) described preneoplastic lesions of various types in addition to tumours of the liver

(mainly hepatocellular carcinomas) in rodents (rats and Syrian hamsters) and non-human primates (rhesus, cynomolgus and African green monkeys). There have been numerous subsequent studies in aflatoxin B_1-exposed animals, especially rats, of GSTP-positive foci in the liver. Particular emphasis has been placed on modification by different co-exposures of the development and frequency of these foci. Often, there was an increase in the number of GSTP-positive foci by L-buthionine sulfoximine (which depletes reduced glutathione) and inhibition of their appearance or rate of development by phenobarbital, anti-oxidants and various sulfur compounds, including dithiolethiones (e.g., Bolton *et al*., 1993; Gopalan *et al*., 1993; Maxuitenko *et al*., 1993; Primiano *et al*., 1995; Hiruma *et al*., 1996; Williams & Iatropoulos, 1996; Hiruma *et al*., 1997; Soni *et al*., 1997; Maxuitenko *et al*., 1998).

(*a*) *Immunosuppression*

Studies on the immunosuppressive effects of aflatoxins published before 1993 were reviewed in the previous monograph (IARC, 1993).

Aflatoxins modulate the immune system in domestic and laboratory animals after dietary intake of up to several milligrams per kg feed (Hall & Wild, 1994; Bondy & Pestka, 2000). The major effects involve suppression of cell-mediated immunity, most notably impairment of delayed-type hypersensitivity, which has been a consistent observation at low dose levels in various species (Bondy & Pestka, 2000). Other notable effects include suppression of non-specific humoral substances, reduced antibody formation, suppression of allograft rejection, decreased phagocytic activity and decreased blastogenic response to mitogens (Pier & McLoughlin, 1985; Denning, 1987; WHO, 1990). Strong modification of cytokine secretion and interleukin gene expression has also been observed *in vitro* with mycotoxins, including aflatoxins (Han *et al*., 1999; Moon *et al*., 1999; Rossano *et al*., 1999). The immune system of developing pigs was affected by maternal dietary exposure to aflatoxin B_1 or aflatoxin G_1 during gestation and lactation. Motility and chemotaxis of neutrophils were inhibited in piglets from aflatoxin-treated sows (Silvotti *et al*., 1997). In a further study, thymic cortical lymphocytes were depleted and thymus weight was reduced in piglets from sows exposed to aflatoxin B_1 (800 ppb [µg/kg] in diet) from day 60 of gestation up to day 28 of lactation (Mocchegiani *et al*., 1998).

The effects of aflatoxin B_1 on growing rats have been shown to be similar to those in adult animals. Weanling rats [strain unspecified] were given oral doses of 60, 300 or 600 µg/kg bw aflatoxin B_1 in corn oil every other day for four weeks. Aflatoxin B_1 selectively suppressed cell-mediated immunity, assessed by measuring the delayed-type hypersensitivity response, at the 300- and 600-µg/kg bw doses (Raisuddin *et al*., 1993).

In order to determine the effect of aflatoxin B_1 on the activation of toxoplasmosis, CF1 mice were injected with the cyst-forming parasite *Toxoplasma gondii* one month before aflatoxin B_1 was given by gavage daily for 50 days at 100 µg/kg bw. Cysts developed in the brains of all mice, but the lesions were judged to be more severe in the aflatoxin B_1-treated animals (Venturini *et al*., 1996).

Several studies have been reported on the effect of aflatoxin on isolated alveolar macrophages, but only few experiments in intact animals. One such study involved male Fischer 344 rats and female Swiss mice that were exposed to aflatoxin B_1 by either aerosol inhalation or intratracheal instillation. Nose-only inhalation exposure of rats to aflatoxin B_1 aerosols suppressed alveolar macrophage phagocytosis at an estimated dose of 16.8 µg/kg bw. The effect persisted for about two weeks. The effects after intratracheal exposures were similar but occurred at approximately 10-fold higher doses. Additionally, intratracheal administration of aflatoxin B_1 suppressed the release of tumour necrosis factor α (TNFα) and inhibited peritoneal macrophage phagocytosis (Jakab *et al.*, 1994).

The overall picture from studies of immunosuppressive effects of aflatoxins in animals is of increased susceptibility to bacterial and parasitic infections and an adverse effect on acquired immunity, as evidenced by experimental challenge with infectious agents after vaccination (reviewed by Denning, 1987). In contrast to the evidence of the immunosuppressive action of aflatoxins in animal studies, evidence in humans comes only from in-vitro experiments. Extremely low doses of aflatoxin B_1 (0.5–1.0 pg/mL) in cultures of human monocytes *in vitro* were shown to decrease phagocytosis and microbicidal activity against *Candida albicans* (Cusumano *et al*., 1996). Concentrations as low as 0.05 pg/mL were shown to reduce the release of interleukins 1 and 6 and TNFα (Rossano *et al.*, 1999). Mycotoxin-induced immune disruption may influence susceptibility to childhood infections, but may also increase later susceptibility to hepatocellular carcinoma through the child's reduced immune response to hepatitis B virus (HBV) and risk of subsequent development of chronic HBV-carrier status (see Section 4.5.3).

4.3 Reproductive and developmental effects

Reproductive and developmental effects of aflatoxins were reviewed in the previous monograph (IARC, 1993). Aflatoxins cross the placental barrier, and there is some evidence that concentrations in cord blood are higher than those in maternal blood (Lamplugh *et al.*, 1988). Malformations and reduced fetal weight have been found in mice administered high doses (32–90 mg/kg bw) of aflatoxin intraperitoneally. No corresponding effect was seen after oral treatment (Tanimura *et al.*, 1982; Roll *et al.*, 1990). In rats, decreased fetal weight and behavioural changes, but not malformations, have been found at dose levels of around 2–7 mg/kg bw (Sharma & Sahai, 1987).

4.3.1 *Humans*

Several studies have reported high levels of free aflatoxins in maternal and umbilical cord blood in humans living in areas where consumption of large amounts of food highly contaminated with aflatoxins is suspected or has been demonstrated in previous studies. However, the chemical analysis in each study relied on a single method and the results were not confirmed by other means. A number of studies have reported effects in infants,

but in most studies, various confounders were not controlled for and exposure levels were not investigated.

Aflatoxins have been reported to occur in up to 40% of samples of breast milk collected from women in tropical Africa (Hendrickse, 1997) (see also Section 1.3.3(*b*)).

Concentrations of aflatoxin M_1 were measured in breast milk of women from Victoria (Australia) and Thailand as a biomarker for exposure to aflatoxin B_1. Aflatoxin M_1 was detected in 11 of 73 samples from Victoria (median concentration, 0.071 ng/mL) and in five of 11 samples from Thailand (median concentration, 0.664 ng/mL) (El-Nezami *et al.*, 1995).

In a survey of the occurrence of aflatoxins in mothers' breast milk carried out in Abu Dhabi and involving 445 donors, 99.5% of samples contained concentrations of aflatoxin M_1 ranging from 2 pg/mL to 3 ng/mL (Saad *et al*., 1995).

Maxwell (1998) reviewed the presence of aflatoxins in human body fluids and tissues in relation to child health in the tropics. In Ghana, Kenya, Nigeria and Sierra Leone, 25% of cord blood samples contained aflatoxins, primarily M_1 and M_2, in variable amounts (range for aflatoxin M_1: 7 ng/L–65 µg/L).

Of 35 cord serum samples from Thailand, 17 (48%) contained aflatoxin concentrations of 0.064–13.6 nmol/mL (mean, 3.1 nmol/mL). By comparison, only two (6%) of 35 maternal sera obtained immediately after birth of the child contained aflatoxin (mean, 0.62 nmol/mL). These results demonstrate transplacental transfer and indicate that aflatoxin is concentrated by the feto-placental unit (Denning *et al*., 1990).

A study of 480 children (aged 1–5 years) in Benin and Togo examined aflatoxin exposure in relation to growth parameters. Mean concentrations of aflatoxin–albumin adducts in the blood were 2.5-fold higher in fully weaned children than in those who were still partially breast-fed. There was a strong negative correlation between aflatoxin–albumin adduct levels in the blood and both height-for-age (stunting) and weight-for-age (being underweight) compared with WHO reference population data after adjustment for age, sex, weaning status, socioeconomic status and geographical location. These data suggest that aflatoxin may inhibit growth in West African children (Gong *et al.*, 2002).

In a small study of the presence of aflatoxin in cord blood in Ibadan, Nigeria, a significant reduction in birth weight was found in jaundiced neonates, who had significantly higher serum aflatoxin concentrations compared with babies without jaundice (Abulu *et al*., 1998).

In a study to investigate whether aflatoxins contribute to the occurrence of jaundice in Ibadan, blood samples were obtained from 327 jaundiced neonates and 60 non-jaundiced controls. Aflatoxins were detected in 24.7% of jaundiced neonates and in 16.6% of controls. Analysis of the data indicated that either glucose-6-phosphate dehydrogenase deficiency or serum aflatoxin are risk factors for neonatal jaundice; odds ratios were significantly increased: 3.0 (95% CI, 1.3–6.7) and 2.7 (95% CI, 1.2–6.1), respectively (Sodeinde *et al*., 1995).

Aflatoxins were detected in 14 of 64 (37.8%) cord blood samples from jaundiced neonates compared with 9 of 60 (22.5%) samples from non-jaundiced control babies in another study in Nigeria, but the difference was not statistically significant (Ahmed *et al*., 1995).

Aflatoxins were detected in 37% of cord blood samples in a study of 125 pregnancies in rural Kenya, with 53% of maternal blood samples being aflatoxin-positive. There was no correlation between aflatoxins in maternal and cord blood. A significantly lower mean birth weight of infants born to aflatoxin-positive mothers was recorded for female babies, but not for males (De Vries *et al*., 1989).

In cord blood collected from 625 babies in Nigeria, aflatoxins were detected in 14.6% of the samples. There was no significant difference in birth weight between the groups positive or negative for aflatoxins (Maxwell *et al*., 1994).

In a study of the presence of the imidazole ring-opened form of aflatoxin B_1–DNA adducts (see Figure 3) in placenta and cord blood, 69 of 120 (57.5%) placentas contained the adduct at 0.6–6.3 μmol/mol DNA and 5 of 56 (8.9%) cord blood samples contained the adduct at 1.4–2.7 μmol/mol DNA. The results indicate that transplacental transfer of aflatoxin B_1 and its metabolites to the progeny is possible (Hsieh & Hsieh, 1993).

A random sampling of semen from adult men, comprising 50 samples collected from infertile men and 50 samples from fertile men from the same community in Nigeria, revealed the presence of aflatoxin B_1 in 40% of samples from infertile men compared with 8% in fertile men. The mean concentration of aflatoxins in semen of the infertile men was significantly higher than that in semen of fertile men. Infertile men with aflatoxin in their semen showed a higher percentage of spermatozoal abnormalities (50%) than the fertile men (10–15%) (Ibeh *et al*., 1994).

4.3.2 *Experimental systems*

(*a*) *Developmental toxicity studies*

Behavioural effects were observed in offspring born to Jcl:Wistar rats given subcutaneous injections of 0.3 mg/kg bw aflatoxin B_1 per day on gestation days 11–14 or 15–18. At birth, the number of live pups and their body weight were lower than those of controls. There were no effects on maternal body weight during gestation or lactation. The exposure produced a delay in early response development, impaired locomotor coordination and impaired learning ability. Exposure on days 11–14 of gestation appeared to produce more effects than later exposure (Kihara et al., 2000).

Aflatoxin B_1 produced embryonic mortality and decreased embryo weight and length when injected into embryonating chicken eggs. The number of abnormal embryos was not significantly increased (Edrington *et al*., 1995).

(*b*) *Reproductive toxicity studies*

Effects suggesting severe impairment of fertility, i.e., reductions in ovarian and uterine size, increases in fetal resorption, disturbances of estrus cyclicity, inhibition of

lordosis and reduction in conception rates and litter sizes, were observed in Druckrey rats exposed to 7.5 mg/kg bw aflatoxin B_1 per day for 14 days. An aflatoxin B_1 blood concentration of 86.2 [μg/L] ppb was found in the exposed animals (Ibeh & Saxena, 1997a).

Female Druckrey rats were given oral doses of 7.5 or 15 mg/kg bw aflatoxin B_1 daily for 21 days. Dose-dependent reductions were seen in the number of oocytes and large follicles. The blood hormone levels and sex organ weight were also disturbed (Ibeh & Saxena, 1997b).

Male mature rabbits were given oral doses of 15 or 30 μg/kg bw aflatoxin B_1 every other day for nine weeks followed by a nine-week recovery period. Body weight, relative testes weight, serum testosterone, ejaculate volume, sperm concentration and sperm motility were reduced and the number of abnormal sperm was increased in a dose-dependent manner. These effects continued during the recovery period. Simultaneous treatment with ascorbic acid (20 mg/kg bw) alleviated the effects of exposure to aflatoxin B_1 during the treatment and recovery period (Salem *et al.*, 2001).

The reproductive performance of female mink (Mustela vison) given a diet containing 5 or 10 ppb [μg/kg] total aflatoxins from naturally contaminated corn for 90 days was not impaired compared with a control group. Body weights of the kits were significantly decreased at the 10-ppb dose at birth and in both exposed groups at three weeks of age. Kit mortality was highest in the 10-ppb group and reached 33% by three weeks of age. In the 10-ppb dose group, analysis of milk samples showed very low concentrations of aflatoxin metabolites (Aulerich *et al.*, 1993).

In an experiment to determine the effects of aflatoxin B_1 (2–16 ppb [μg/L] in medium) on the in-vitro fertilizing ability of oocytes and epididymal sperm of albino rats, a significant reduction of the mean number of oocytes fertilized was observed, as well as a significant decrease in sperm motility (Ibeh *et al.*, 2000).

4.4 Genetic and related effects

4.4.1 *Humans*

(*a*) *General*

DNA and protein adducts of aflatoxin have been detected in many studies of human liver tissues and body fluids (IARC, 1993). Some studies related the level of adducts detected to polymorphisms in metabolizing enzymes, in order to investigate interindividual susceptibility to aflatoxin.

Wild *et al*. (1993b) measured serum aflatoxin–albumin adducts in 117 Gambian children in relation to *GSTM1* genotype and found no difference in adduct levels by genotype.

In a larger study of 357 adults in the same population, aflatoxin–albumin adduct levels were examined in relation to genetic polymorphisms in the *GSTM1*, *GSTT1*, *GSTP1* and epoxide hydrolase genes. Only the *GSTM1*-null genotype was associated with a modest increase in aflatoxin–albumin adduct levels and this effect was restricted

to non-HBV-infected individuals. *CYP3A4* phenotype, as judged by urinary cortisol metabolite ratios, was also not associated with adduct level. The main factors affecting the level of aflatoxin–albumin adducts were place of residence (rural areas higher than urban areas) and season of blood sample collection (dry season higher than wet season) (Wild *et al.*, 2000). Kensler *et al*. (1998) also found no association between aflatoxin–albumin adducts and *GSTM1* genotype in 234 adults from Qidong County, China.

The role of polymorphisms in the DNA repair enzyme, XRCC1, in influencing the levels of aflatoxin B_1–DNA adducts in samples of placental DNA was studied in women at a Taiwanese maternity clinic. The presence of at least one allele of polymorphism, 399Gln, was associated with a two- to three-fold higher risk of having detectable aflatoxin B_1–DNA adducts compared with subjects homozygous for the 399Arg allele. However, when the association between polymorphism and tertiles of adduct level was examined, the 399Gln allele was associated with intermediate but not high adduct levels. The authors suggested that this may reflect saturation of repair pathways (Lunn *et al.*, 1999).

Studies of the types of genetic alteration associated with exposure to aflatoxin *in vivo* have been less extensive. In human subjects from Qidong County, China, aflatoxin exposure was determined as high or low (dichotomized around the population mean) by aflatoxin–albumin adduct level in serum and compared with the *HPRT* mutation frequency in lymphocytes. A raised *HPRT* mutant frequency was observed in subjects with high compared with low aflatoxin exposure (OR, 19; 95% CI, 2.0–183) (Wang *et al*., 1999b).

The levels of chromosomal aberrations, micronuclei and sister chromatid exchange were studied in 35 Gambian adults, 32 of whom had measurable concentrations of aflatoxin–albumin adducts. There was no correlation within this group between the cytogenetic alterations and aflatoxin–albumin adducts in peripheral blood at the individual level. In a further study, blood samples of 29 individuals of the same Gambian group were tested for DNA damage in the single-cell gel electrophoresis (comet) assay but no correlation was observed with aflatoxin–albumin adducts or *GSTM1* genotype (Anderson *et al*., 1999).

(*b*) *TP53 mutations in human hepatocellular carcinoma (HCC)*

Molecular analyses of human HCC have revealed a high prevalence of an AGG to AGT (arg to ser) transversion at codon 249 of the *TP53* tumour-suppressor gene (249^{ser} mutation) in tumours from areas of the world with reported high aflatoxin exposure (Montesano *et al*., 1997). A large number of studies have been published since 1993 on aflatoxin exposure and *TP53* mutations; two recent meta-analyses examined the relationship between aflatoxin exposure, HBV infection and *TP53* mutations in 20 (Lasky & Magder, 1997) and in 48 published studies (Stern *et al*., 2001). Table 15 summarizes the published data and the key findings are described below. [It is important to note that the specificity of *TP53* mutations in relation to aflatoxin exposure is associated only with G to T transversions at the third base of codon 249, whereas the meta-analysis by Stern *et al.* (2001) included a few G to T transversions in the second base of

Table 15. Analyses of *TP53* codon 249^{ser} mutations in human hepatocellular carcinomas (HCC)

Region/country	No. of HCC analysed	No. with codon 249^{ser} mutation	Reference
Africa			
Mozambique	15	8	Ozturk (1991)
South Africa — Transkei	12	1	Ozturk (1991)
Southern Africa	10	3	Bressac *et al*. (1991)
Senegal	15	10	Coursaget *et al*. (1993)
America			
USA			
Alaska	7	0	Buetow *et al*. (1992)
Alaskans	12	0	De Benedetti *et al*. (1995)
	12	0	Kazachkov *et al*. (1996)
	17	0	Wong *et al*. (2000)
Mexico	16	3	Soini *et al*. (1996)
Asia			
China			
Qidong	36	21	Scorsone *et al*. (1992)
	25	13	Fujimoto *et al*. (1994)
	20	9	Li *et al*. (1993)
Xian	45	1	Buetow *et al*. (1992)
Beijing	9	0	Fujimoto *et al*. (1994)
Tongan	21	7	Yang *et al*. (1997)
Jiang-su south	16	9	Shimizu *et al*. (1999)
Jiang-su north	15	1	Shimizu *et al*. (1999)
Shanghai	12	1	Buetow *et al*. (1992)
	18	1	Li *et al*. (1993)
	20	4	Wong *et al*. (2000)
Guanxi	50	18	Stern *et al*. (2001)
Hong Kong	26	1	Ng *et al*. (1994a,b)
	30	4	Wong *et al*. (2000)
India	21	2	Katiyar *et al*. (2000)
Indonesia	4	1	Oda *et al*. (1992)
Japan	128	1	Oda *et al*. (1992)
	10	0	Buetow *et al*. (1992)
	43	0	Murakami *et al*. (1991)
	60	0	Hayashi *et al*. (1993)
	52	0	Konishi *et al*. (1993)
	53	0	Nishida *et al*. (1993)
	20	0	Nose *et al*. (1993)
	34	3	Tanaka *et al*. (1993)
	41	0	Teramoto *et al*. (1994)
	41	0	Hsieh & Atkinson (1995)
	16	0	Wong *et al*. (2000)

Table 15 (contd)

Region/country	No. of HCC analysed	No. with codon 249^{ser} mutation	Reference
Korea (Republic of)	6	0	Oda *et al*. (1992)
	35	0	Park *et al*. (2000)
Singapore (Chinese)	44	0	Shi *et al*. (1995)
Taiwan, China	2	0	Oda *et al*. (1992)
	12	0	Hosono *et al*. (1993)
Europe			
France	100	2	Laurent-Puig *et al*. (2001)
Germany	13	0	Kress *et al*. (1992)
	20	0	Kubicka *et al*. (1995)
Italy	20	0	Bourdon *et al*. (1995)
Spain	70	0	Boix-Ferrero *et al*. (1999)
United Kingdom	19	0	Challen *et al*. (1992)
	170	0	Vautier *et al*. (1999)

Adapted from Stern *et al.* (2001)

this codon. The authors of the meta-analyses pointed out that many studies, particularly the earlier ones, looked only for the presence or absence of the specific codon 249^{ser} mutation and as a consequence may have overemphasized the importance of this particular mutation among the total of *TP53* mutations in HCC.]

The vast majority of these studies have not directly assessed either population exposure or individual exposure to aflatoxin in relation to *TP53* mutations. Instead, estimates were made from data on aflatoxin levels in food, frequency of consumption of those foods and extrapolation to expected aflatoxin levels based on climatic conditions likely to promote aflatoxin production. This limits the interpretation of these data. Montesano *et al*. (1997) attempted to use biomarker data on human exposure to provide information additional to the estimates based on geographical origin of the samples.

Fujimoto *et al*. (1994) studied 25 HCC tissue samples from Qidong County, China, an area of high aflatoxin exposure, and nine HCC samples from Beijing with lower aflatoxin exposure. Thirteen of 25 tumours (52%) from Qidong carried the 249^{ser} mutation, while none of the nine from Beijing did. Shimizu *et al*. (1999) studied 31 HCC tissue samples from different parts of the Jiang-su province. In the northern region of this province, where aflatoxin exposure is expected to be lower, one of 15 (8%) tumours showed a 249^{ser} mutation, while in the southern part, including Haimen City, nine of 16 (56%) HCC samples showed this mutation. Scorsone *et al*. (1992) found the 249^{ser} mutation in 21 of 36 (58%) of HCC tumour tissue samples from Qidong. In contrast, of 26 HCC examined in Hong Kong, only one (4%) had the specific 249^{ser} mutation, although another tumour had a G to T transversion at the second nucleotide (Ng *et al*., 1994a,b). Wong *et al*. (2000) found the *TP53* 249^{ser} mutation in 4/30 (13%) HCC

samples from Hong Kong and in 4/20 (20%) from Shanghai. This mutation was not found in 16 samples from Japan and 17 from the USA, although there were other mutations in exon 7 in the Japanese samples.

In areas of expected low aflatoxin exposure (including Japan, Republic of Korea, Europe and North America), the prevalence of codon 249 mutations is extremely low (< 1%) and even those that do occur tend to be at the second nucleotide rather than the third. Oda *et al*. (1992) analysed 140 HCC tissue samples (128 Japanese, six Korean, four Indonesian and two Taiwanese); of these, only one Japanese and one Indonesian showed the specific 249^{ser} mutation. The limited information on residence and ethnicity in many studies has been commented upon (Laskey & Magder, 1997).

Hollstein *et al*. (1993) measured serum aflatoxin–albumin adducts and liver aflatoxin B_1–DNA adducts from 15 Thai patients, but only one had measurable concentrations of albumin adducts in serum. In none of the samples were aflatoxin B_1–DNA adducts found. Only one had the specific 249^{ser} mutation. In another study of 16 HCC cases from Mexico (Soini *et al*., 1996), three tumours contained the 249^{ser} mutation; of these, sera were available for two patients and both contained aflatoxin–albumin adducts. Aflatoxin–albumin adducts were detected in sera from all of a further 14 patients without the mutation in the corresponding HCC.

Since chronic HBV infection is a strong and specific risk factor for HCC and aflatoxin exposure commonly co-occurs with viral infection, it is important to examine whether the 249^{ser} mutation is seen only in the presence of chronic HBV infection. Although it is clear from the studies summarized below of HCC in North America, Europe and Japan that HBV alone does not induce the 249^{ser} mutation, the high prevalence of HBV infection in aflatoxin-endemic areas has made it hard to establish whether both risk factors are required for the mutation to occur.

Lasky and Madger (1997) summarized thirteen studies that both ascertained HBV status and analysed *TP53* mutations. Data were available on 449 patients, of whom 201 were positive and 248 negative for HBV markers. The association between aflatoxin exposure and the 249^{ser} mutation was still observed when the analysis was restricted to HBV-positive patients in the groups with high and low aflatoxin exposure. However, the number of HBV-negative patients with high aflatoxin exposure was too small to allow a similar comparison in HBV-negative cases. Overall, it appears that the 249^{ser} mutation related to aflatoxin exposure is not explained by any confounding introduced by possible associations between aflatoxin and HBV.

It remains unclear at what stage in the natural history of HCC the *TP53* mutation occurs. Some information is available from the analysis of histologically normal liver samples from patients resident in areas reportedly differing in aflatoxin exposure level. Aguilar *et al*. (1993, 1994) examined non-tumorous liver tissue from small numbers of HCC patients from Qidong (China), Thailand and the USA and demonstrated the presence of *TP53* AGG to AGT mutations (in codon 249) at a higher frequency in samples from China than in those from Thailand or the USA. By use of an allele-specific polymerase chain reaction (PCR) assay, Kirby *et al.* (1996b) detected the *TP53* 249^{ser}

mutation in non-tumorous liver DNA from five of six HCC patients in Mozambique; none of seven samples from North American patients had a positive signal in the assay. These observations suggest that this type of mutation is found in histologically normal cells of patients with HCC.

The *TP53* 249^{ser} mutation has also been detected in blood samples from HCC patients, patients with cirrhosis and individuals without clinically diagnosed liver disease. Kirk *et al*. (2000) compared 53 HCC patients, 13 patients with cirrhosis and 53 control subjects in The Gambia with 60 non-African French patients, 50 of whom had HCC and 10 had cirrhosis. The 249^{ser} mutation was detected by restriction fragment length polymorphism (RFLP)-PCR in circulating DNA in plasma from 19 (36%) of the HCC patients, two (15%) cirrhosis patients and three (6%) of the African control subjects. The prevalence of the 249^{ser} mutation did not differ between HBsAg-positive and -negative individuals. The mutation was not detected in any of the French plasma samples. No tumour tissue was available from the Gambian patients and so the presence of the same mutations in the corresponding HCC could not be confirmed. The detection of the 249^{ser} mutation in circulating DNA in the plasma of non-cancer patients again could reflect either an early neoplastic event or exposure to aflatoxin.

Jackson *et al*. (2001) examined 20 paired plasma and HCC samples from patients from Qidong County, China, for the presence of the *TP53* 249^{ser} mutation analysed by short oligonucleotide mass spectrometry. Eleven tumours were positive for the mutation and the same mutation was detected in six of the paired plasma samples. An additional four plasma samples were positive for the mutation in the absence of a detectable mutation in the corresponding tumour. The authors suggested that this might be due to other non-sampled HCCs in those patients. In contrast to the findings of Aguilar *et al*. (1993), no 249^{ser} mutations were detected in DNA from normal tissue adjacent to the HCC.

(*c*) *Other genetic alterations in human HCC*

It would be unexpected if aflatoxin carcinogenesis were exclusively associated with a specific *TP53* mutation, given the multiple genetic alterations observed in human HCC. Consequently, several studies have tested the hypothesis that aflatoxin exposure is associated with other specific genetic alterations.

In the study by Fujimoto *et al*. (1994) described above, while the 249^{ser} mutation was more frequent in samples from Qidong than in those from Beijing, additional differences were found in the pattern of loss of heterozygosity (LOH). Specifically, in tumours from Qidong, four of 14 informative cases (28%) showed LOH on chromosome 4 (4p11-q21) and nine of 10 (90%) and 11 of 19 (58%) showed LOH on chromosome 16q22.1 and 16q22-24, respectively. In contrast, none of six informative cases from Beijing showed LOH at 16q22-24 and none of five at 4p11-q21.

Wong *et al*. (2000) studied 83 HCC samples from patients undergoing curative resection. Of these, 50 were from China (30 from Hong Kong, 20 from Shanghai), 16 from Japan and 17 from the USA. The Chinese subjects were all HBV-positive, the Japanese patients were HCV-positive and the patients from the USA were HBV-negative.

In eight subjects (four from Hong Kong and four from Shanghai), single-strand conformation polymorphism (SSCP) analysis and DNA sequencing of exons 5 to 9 of the *TP53* gene revealed the 249^{ser} mutation. However, the authors also performed comparative genomic hybridization. In HCC from Shanghai, there were significantly more alterations per sample in these HBV-related cases than in those from Hong Kong or in the HCCs from Japan and the USA; approximately double the number of alterations per sample was observed in Shanghai compared with Hong Kong. The most frequent changes responsible for this increase were deletions on chromosomes 4q, 8p and 16q and gain of 5p. The authors suggested that this might reflect broader genetic effects of aflatoxin than simply the 249^{ser} mutation in the *TP53* gene.

These studies show that, in addition to *TP53* mutations, geographical location may influence other genetic alterations in HCC, but the data are insufficient to ascribe any of these specifically to aflatoxin exposure.

4.4.2 *Experimental systems*

(*a*) *General*

Aflatoxin B_1 induces mutations in *Salmonella typhimurium* strains TA98 and TA100, and unscheduled DNA synthesis, chromosomal aberrations, sister chromatid exchange, micronucleus formation and cell transformation in various in-vivo and in-vitro mammalian systems (IARC, 1993; for references and details on results published since 1993, see Table 16).

Aflatoxin B_1 can induce mitotic recombination in addition to point mutations. This has been demonstrated in both yeast and mammalian cells. In human lymphoblastoid cells, aflatoxin B_1 treatment led to mitotic recombination and LOH. A reversion assay demonstrated aflatoxin B_1-induced intrachromosomal recombination in a mutant cell line derived from V79 cells harbouring an inactivating tandem duplication in the *Hprt* gene.

Aflatoxin B_1 also induced recombination in minisatellite sequences in yeast expressing recombinant human CYP1A2. In addition, liver tumours derived from HBV-transgenic mice treated with aflatoxin B_1 transplacentally contained rearrangements in minisatellite sequences, but no such alterations were observed in tumours from HBV-transgenic mice not exposed to aflatoxin B_1 (Kaplanski *et al.*, 1997). This suggests that aflatoxin can promote genetic instability in addition to point mutations. Mitotic recombination and genetic instability may therefore be alternative mechanisms by which aflatoxin contributes to genetic alterations such as LOH in HCC (see Section 4.4.1(*c*)).

As expected, aflatoxin B_1 is significantly more mutagenic following metabolic activation. The mutagenicity of aflatoxin B_1 in *Salmonella* tester strains TA98 and TA100 without S9 was approximately 1000 times lower than in the presence of S9.

Splenic lymphocytes were examined for mutant frequency at the *Hprt* locus in Fischer 344 rats exposed to aflatoxin B_1. *Hprt* mutants (frequency, $19.4–31.0 \times 10^{-6}$) were induced after a three-week exposure of male Fischer 344 rats to aflatoxin B_1 by repeated intragastric dosing to a total dose of 1500 μg/kg bw. In the same experiment,

Table 16. Genetic and related effects of aflatoxin B_1

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Salmonella typhimurium TA100, TA98, reverse mutation	NT	+	2.5 ng/tube	Loarca-Piña *et al*. (1996)
Salmonella typhimurium TA100, TA98, reverse mutation	+	NT	0.6 µg/tube	Loarca-Piña *et al*. (1998)
Salmonella typhimurium TA98, reverse mutation (S9 mix from HepG2 cells)	NT	+	2 µg/plate	Knasmüller *et al*. (1998)
Saccharomyces cerevisiae with recombinant CYP1A2 (human), mitotic recombination	+	NT	5	Kaplanski *et al*. (1998)
DNA damage (comet assay), human HepG2 cells *in vitro*	+		0.0025	Uhl *et al*. (2000)
Gene mutation, human hepatoma (HepG2) cells *in vitro*, *HPRT* locus	NT	+	0.5	Knasmüller *et al*. (1998)
Gene mutation, Chinese hamster ovary AS52 cells *in vitro*, *Gpt* locus	NT	+	0.16	Goeger *et al*. (1998)
Gene mutation, Chinese hamster ovary K_1BH_4 cells *in vitro*, *Hprt* locus	NT	+	0.31	Goeger *et al*. (1998)
Gene mutation, Chinese hamster ovary K_1BH_4 cells *in vitro*, *Hprt* locus	NT	+	0.31	Goeger *et al*. (1999)
Gene mutation, mouse lymphoma L5178Y cells *in vitro*, *tk* locus	NT	+[d]	0.005	Preisler *et al*. (2000)
Recombination, yeast *S. cerevisiae in vitro*[c]	+	NT	7.8	Sengstag *et al*. (1996)
Recombination, Chinese hamster V79 SP5 cells *in vitro*, reversion mutation assay	NT	+	0.19	Zhang & Jenssen (1994)
Micronucleus formation, Chinese hamster V97MZr2B1 cells *in vitro*[e]	+	NT	0.031	Reen *et al*. (1997)
Cell transformation, rat liver epithelial BL9 cells	–	+	7.5	Stanley *et al*. (1999)
Gene mutation, human lymphoblastoid cells (recombinant CYP1A1) *in vitro*, *HPRT* locus	+	NT	0.004	Cariello *et al*. (1994)
Recombination, human lymphoblastoid TK6 cells *in vitro*, *TK* locus	NT	+	0.016	Stettler & Sengstag (2001)
Sister chromatid exchange, human lymphocytes *in vitro*	+	+	9.4	Wilson *et al*. (1995)
Sister chromatid exchange, human leukocytes *in vitro*	NT	+[f]	0.31	Wilson *et al*. (1997)

Table 16 (contd)

Test system	Result[a]		Dose[b] (LED or HID)	Reference
	Without exogenous metabolic system	With exogenous metabolic system		
Gene mutation, male F344 rat splenic lymphocytes *in vivo*, *Hprt* locus	+		0.1 on 5 d/w for 3 w, po	Casciano *et al*. (1996)
Gene mutation, male F344 rat splenic lymphocytes *in vivo*, *Hprt* locus	+		0.01 ppm in diet (inter-mittent)[g]	Morris *et al*. (1999)
Gene mutation, Big Blue male C57BL/6 mice *in vivo*, *LacI* locus	–		2.5 × 1 ip	Dycaico *et al*. (1996)
Gene mutation, Big Blue male Fischer 344 rats *in vivo*, *LacI* locus	+		0.25 × 1 ip	Dycaico *et al*. (1996)
Micronucleus formation, male Swiss mouse bone marrow *in vivo*	–		1.0 ip × 1	Anwar *et al*. (1994)
Micronucleus formation, male Wistar rat bone marrow *in vivo*	+		0.1 ip × 1	Anwar *et al*. (1994)
Chromosomal aberrations, male Swiss mouse bone marrow *in vivo*	(+)		1.0 ip × 1	Anwar *et al*. (1994)
Chromosomal aberrations, male Wistar rat bone marrow *in vivo*	+		0.1 ip × 1	Anwar *et al*. (1994)
Gene mutation, intrasanguineous host-mediated assay (Wistar rat), *E. coli* K12, *LacI* locus	+		1 ip × 1	Prieto-Alamo *et al*. (1996)

[a] +, positive; (+), weak positive; –, negative; ?, inconclusive; NT, not tested

[b] LED, lowest effective dose; HID, highest ineffective dose; in-vitro tests, μg/mL; in-vivo tests, mg/kg bw/day; NG, not given; po, oral; im, intramuscular; ip, intraperitoneal

[c] Transfected with cDNA encoding human CYP1A1 or CYP1A2

[d] Majority of mutants due to mitotic recombination

[e] Transfected with cDNA encoding rat liver CYP2B1

[f] Activation by microsomes from mouse liver; those from rat or human liver were less active; presence of cytosol from mouse reduced activity, but rat or human cytosol did not.

[g] Animals received aflatoxin B_1 during alternating four-week periods (5–8, 13–16 and 21–24 weeks of age).

aflatoxin-treated rats on a calorically restricted diet showed much lower mutation frequencies (Casciano *et al.*, 1996).

In an intermittent feeding trial, Fischer 344 rats (aged four weeks at the start of the trial) were exposed either to (*a*) control diet, (*b*) various concentrations of aflatoxin B_1 (0.01, 0.10, 0.04, 0.4 or 1.6 ppm [mg/kg]) in the diet for three four-week periods separated by four-week periods of control diet or (*c*) a tumorigenic dose of aflatoxin B_1 (1.6 ppm) in the diet continuously for 20 weeks. A dose-dependent increase in the *Hprt* mutant frequency in splenic lymphocytes was observed after the second four-week feeding period. This effect was further enhanced after the third four-week feeding period, at which time a particularly strong response was observed with the 0.4-ppm dose (mean mutant frequency $> 70 \times 10^{-6}$). These results may be explained by accumulation of DNA damage in splenic lymphocyte DNA (Morris *et al.*, 1999).

The species differences in susceptibility to the toxic and carcinogenic effects of aflatoxins (Section 4.1.2) are reflected in the differing activities of microsomal preparations to produce genetic damage following aflatoxin treatment. Human, mouse and rat liver preparations were used to activate aflatoxin B_1 and the induction of sister chromatid exchange in human mononuclear leukocytes was examined. In leukocytes treated with aflatoxin B_1 activated by human liver microsomes from six different donors, there was a 10-fold interindividual variation in the mean number (1.1–11.6) of sister chromatid exchanges per cell. The induction of sister chromatid exchange was correlated with CYP1A2 phenotype (using model substrates) in the same livers but not with *GSTM1* genotype or epoxide hydrolase phenotype. Mouse microsomes were more effective than rat or human at activating aflatoxin B_1 to induce sister chromatid exchange. The addition of mouse but not human or rat liver cytosol reduced aflatoxin B_1-induced genotoxicity (Wilson *et al.*, 1997).

Mutation assays of the xanthine-guanine phosphoribosyltransferase (*Gpt*) gene in Chinese hamster ovary AS52 cells and at the *Hprt* gene in Chinese hamster ovary K_1BH_4 cells were performed with aflatoxin B_1 metabolized by liver S9 either from chick embryos or rats; the effect of coumarin as a chemoprotectant was also examined. In the *Gpt* assay, 1 μM aflatoxin B_1 induced 25-fold more mutants when chick S9 was used than with rat S9. Coumarin (50 and 500 μM) decreased the mutant frequency by 52 and 88% with the chick embryo-activated aflatoxin B_1 but had no effect on the frequency following activation with rat S9. In K_1BH_4 cells, a dose of 1 μM aflatoxin B_1 induced approximately sixfold more *Hprt* mutants per 10^6 clonable cells when activated by chick embryo than by rat liver S9 (Goeger *et al.*, 1998).

Male rats and mice were treated with single doses of aflatoxin B_1 (0.01–1.0 mg/kg bw) and the frequency of chromosomal aberrations and micronuclei in bone marrow and the amount of aflatoxin B_1–albumin adducts in peripheral blood were measured. In rats, both chromosomal aberrations and micronuclei showed increased frequency at doses above 0.1 mg/kg, whereas in mice only a slight increase in chromosomal aberrations was seen with the highest dose (1.0 mg/kg) and no effect on micronuclei was detected. In rats,

aflatoxin B_1–albumin adduct levels were correlated with chromosomal aberrations at the individual level (Anwar *et al*., 1994).

Mutations *in vivo* were also studied in lambda/*lacI* (Big Blue®) transgenic C57BL/6 mice and Fischer 344 rats treated with aflatoxin B_1. Six mice were given a single intraperitoneal injection of 2.5 mg/kg bw aflatoxin B_1 but no mutations were detected in the liver 14 days after treatment. In contrast, in six rats treated with 0.25 mg/kg bw aflatoxin B_1, there was a nearly 20-fold increase in mutant frequency (mean, 49×10^{-5}) compared with controls. In mutated *lacI* DNA isolated from rats treated with aflatoxin B_1, the predominant mutations (78%) were GC to TA transversions, compared with 11% of spontaneous mutations of this type in control rats. Of the G to T transversions in the treated rats, 71% were at CpG sites. In particular, 5′-GC<u>G</u>G-3′ and 5′-CC<u>G</u>C-3′ sequences were hotspots (target G underlined) (Dycaico *et al*., 1996).

Transgenic mice were developed carrying both the human *CYP3A7* gene, expressed in the small intestine but not kidney, and the *rsp*L gene from *Escherichia coli* as a target for mutations. Microsomal preparations from the small intestine of these transgenic mice had higher capacity to convert aflatoxin B_1 to a mutagen in the Ames test with *Salmonella typhimurium* TA98 strain than did kidney microsomes from the same mice or small intestine and kidney microsomes from non-transgenic mice. In addition, the target *rsp*L gene in mice carrying the *CYP3A7* transgene contained a significantly higher mutation frequency in the small intestine than kidney or than either organ in mice non-transgenic for *CYP3A7* (Yamada *et al*., 1998).

A number of differences have been reported in metabolic activity of S9 fractions from organs other than liver. Ball *et al*. (1995) compared the ability of tracheal and lung S9 from rabbit (male, New Zealand white), hamster (male Syrian golden) and rat (male Sprague Dawley) to induce aflatoxin B_1 mutations in the TA98 strain of *Salmonella typhimurium*. Trachea from hamster and rabbit and lung from rabbit showed a positive response in the assay. In hamsters, trachea S9 was more efficient than lung S9 in producing aflatoxin B_1-induced mutations, while in rabbits the opposite was true.

(*b*) *TP53 mutations in animal tumours*

In order to test the plausibility of an association between aflatoxin exposure and *TP53* mutations, HCC or preneoplastic lesions from several species have been examined for mutations at the codon corresponding to codon 249 in humans, referred to as ‘codon 249 equivalent’ (Wild & Kleihues, 1996).

In contrast to the specific mutations found in human HCC from areas with high exposure to aflatoxin, no G to T transversions in ‘codon 249 equivalent’ have been identified in animal tumours. Two major limitations to making a valid comparison are the different DNA sequences in this region of the *TP53* gene across species and the relatively few animal tumours analysed. In ducks, for example, the third nucleotide is not a guanine but a cytosine and no mutations were observed at ‘codon 249 equivalent’ (Duflot *et al*., 1994). In all the non-primates studied, codon sequences with any base following CG would lead to a silent mutation at the third nucleotide, each base change resulting in

coding for arginine. In four rhesus monkeys (*Macaca mulatta*) and four cynomolgus monkeys (*M. fascicularis*), aflatoxin-induced HCC did not carry any mutation at codon 249, but only four HCC (two from one animal) were analysed (Fujimoto *et al*., 1992).

Preneoplastic lesions have been examined to define the time point in the natural history of HCC when the *TP53* mutation occurs. Hulla *et al*. (1993) examined six hyperplastic nodules from rat liver following intraperitoneal treatment with 150 µg/kg aflatoxin B_1 for 10 days (2 × 5 days with a two-day break in between, followed by partial hepatectomy three weeks later and sacrifice after a further three weeks) and found no mutations at the codon 249 equivalent.

Female $AC3F_1$ (A/J × C3H/He/J) mice [numbers unspecified], 5–7 weeks of age, received intraperitoneal injections of aflatoxin B_1 three times per week for eight weeks (total dose, 150 mg/kg bw). Mice were killed between 6 and 14 months later. Of the 71 lung tumours examined, 79% showed positive nuclear p53 staining. SSCP analysis of microdissected tumour samples revealed mutations in different codons in exons 5, 6 and 7. Direct sequencing showed 26 mutations which included nine G:C to A:T transitions, 11 A:T to G:C transitions and five transversions (two G:C to T:A, two T:A to A:T and one A:T to C:G). The high mutation frequency and heterogeneous staining pattern suggested that *TP53* mutations occur relatively late in aflatoxin B_1-induced mouse lung tumorigenesis (Tam *et al*., 1999).

Lee *et al*. (1998) treated 10 male Sprague-Dawley rats with 37.5 µg aflatoxin B_1 five times per week for eight weeks by gavage and a further 20 with the same treatment after partial hepatectomy. Of the latter group, 13 of 17 rats that survived to 60 weeks after treatment had either liver tumours (5), preneoplastic nodules (7) or both (1). Of the six surviving rats from the group that received aflatoxin B_1 alone, two had liver tumours, one had liver focal lesions and one had both. All rats were killed at 60 weeks and liver tumours and preneoplastic lesions were excised. A total of 19 abnormal liver specimens were obtained (10 liver focal lesions and nine liver tumours). The PCR SSCP method was used to screen the *TP53* gene; five rat livers (29%) exhibited abnormal conformational polymorphisms, all five being from the group receiving aflatoxin B_1 after partial hepatectomy. No *TP53* alterations were detected in four samples from the group that received aflatoxin B_1 alone. One liver tumour contained a silent mutation at codon 247 (CGG to CGT, Arg to Arg).

Aflatoxin B_1 activated with quail liver microsomes transformed BL9 rat epithelial cells *in vitro* and these transformed cells induced liver tumours in nude mice. However, the tumours did not contain mutations in codons 242–244 of the *TP53* gene (spanning the equivalent to codon 249 in human *TP53*) (Stanley *et al*., 1999).

Tree shrews (*Tupaia belangeri chinensis*), which can be infected with human HBV, were used to study aflatoxin B_1–induced mutations in the presence or absence of viral infection. Park *et al*. (2000) studied eight tree shrews, four infected with HBV at 12 weeks of age and four not infected. Two of the uninfected and all four infected animals were treated with 400 µg/kg bw aflatoxin B_1 per day for six days. Five liver tumours were detected upon sacrifice at age 2–3 years, four from HBV- and aflatoxin-treated

animals and one from one of the two animals receiving aflatoxin B_1 alone. No specific *TP53* mutations were observed in relation to the codon 249 hotspot.

TP53 mutations in extrahepatic tumours induced by aflatoxin have been rarely studied. Two cholangiocarcinomas, a spindle-cell carcinoma of the bile duct, a haemangioendothelial sarcoma of the liver and an osteogenic sarcoma of the tibia from rhesus and cynomolgus monkeys treated with aflatoxin B_1 were analysed (Fujimoto *et al*., 1992), but no codon 249 mutations were found. Lung tumours induced in female $AC3F_1$ mice by 150 mg/kg bw aflatoxin B_1 (divided into 24 doses over eight weeks) were microdissected and *TP53* mutations were shown to be frequent events by SSCP and direct sequencing. Most mutations were base substitutions of different types and these were distributed across exons 5–7 of the gene (Tam *et al*., 1999).

Lung cells isolated from $AC3F_1$ mice seven weeks after treatment with aflatoxin B_1 (2×50 mg/kg bw, given two weeks apart) were examined for point mutations in the Ki-*ras* gene. Ki-*ras* mutant alleles were detected in Clara cells but not in other enriched cell fractions. This result indicates the susceptibility of Clara cells to Ki-*ras* activation, an early event in aflatoxin B_1-induced mouse lung tumorigenesis (Donnelly & Massey, 1999).

(*c*) *Sequence-specific binding to DNA and induction of mutations*

Aflatoxin B_1 is metabolically activated to its 8,9-*exo*-epoxide, which reacts with DNA to form the 8,9-dihydro-8-(*N*7-guanyl)-9-hydroxy aflatoxin B_1 (AFB_1–*N*7-Gua) adduct (see Section 4.1.2). The positively charged imidazole ring of the guanine adduct promotes depurination and consequently, apurinic site formation. Under slightly alkaline conditions, the imidazole ring of AFB_1-*N*7-Gua is opened and forms a more stable and persistent ring-opened aflatoxin B_1–formamidopyrimidine adduct. Investigations have been conducted to establish which is the most likely precursor of the mutations induced by aflatoxin B_1.

The mutations induced by aflatoxin B_1 in a number of experimental systems are certainly consistent with the main carcinogen binding occurring at guanine in DNA, leading to G to T transversions (IARC, 1993). When a pS189 shuttle vector was aflatoxin B_1-modified and then replicated in human Ad293 cells, predominantly G to T transversions were detected (Trottier *et al*., 1992). However, other types of mutation have also been observed with aflatoxin B_1. Levy *et al*. (1992) transfected an aflatoxin B_1-modified shuttle vector into DNA repair-deficient (XP) or -proficient human fibroblasts, and examined mutations in the *supF* marker gene. Higher mutation frequencies were observed in the DNA repair-deficient cells and the location of mutations was significantly affected by repair proficiency. The majority of mutations were at GC base pairs: 50–70% were G to T transversions, but G to C transversions and G to A transitions were also frequent. A polymerase stop assay was used to examine the location of aflatoxin B_1 binding within the shuttle vector, but no strong correlation was found between initial binding sites and subsequent hotspots for mutation. This suggests that the processing of the adducts, e.g.,

during DNA replication and repair, can influence not only the overall mutation frequency but also the distribution of mutations.

An intrasanguineous host-mediated assay was used to determine the pattern of mutagenesis induced by aflatoxin B_1 in the *lacI* gene of *E. coli* bacteria recovered from rat liver. Most of the 281 mutations analysed were base substitutions at GC base pairs; over half were GC to TA transversions, with other mutations evenly divided between GC to AT transitions and GC to CG transversions (Prieto-Alamo *et al*., 1996).

In a human lymphoblastoid cell line (h1A2v2) expressing recombinant human CYP1A1 enzyme, aflatoxin B_1 (4 ng/mL; 25 h) produced a hotspot GC to TA transversion mutation at base pair 209 in exon 3 of the *HPRT* gene in 10–17% of all mutants (Cariello *et al*., 1994). This hotspot occurred at a GGGGGG sequence (target base underlined).

Bailey *et al*. (1996) studied the induction of mutations with two of the principal forms of DNA damage induced by aflatoxin B_1, namely the AFB_1–*N*7-Gua adduct and the consequent apurinic sites, by site-directed mutagenesis. Single-stranded M13 bacteriophage DNA containing a unique AFB_1–*N*7-Gua adduct or an apurinic site was used to transform *E. coli*. The predominant mutations with AFB_1–*N*7-Gua were G to T transversions targeted to the site of the original adduct (approximately 74%), with lower frequencies of G to A transitions (13–18%) and G to C transversions (1–3%). Using *E. coli* strains differing in biochemical activity of UmuDC and MucAB — proteins involved in processing of apurinic sites by insertion of dAMP — the authors showed that the mutations observed with the AFB_1–*N*7-Gua were not predominantly a simple result of depurination of the initial adduct. A significant number of base substitutions were located at the base 5′ to the site of the original adduct, representing around 13% of the total mutations. This induction of mutations at the base adjacent to the original site of damage was not observed with apurinic sites as the mutagenic lesion. This was suggested to reflect interference with DNA replication following the intercalation of aflatoxin B_1 8,9-epoxide (Gopalakrishnan *et al*., 1990).

Earlier studies suggested general sequence preferences for aflatoxin B_1 binding dependent on the target guanine being located in a sequence of guanines or with a 5′ cytosine (IARC, 1993). The base 3′ to the modified G appears less consistently predictive of reactivity. Results on sequence-specific binding have been reviewed (Smela *et al*., 2001). In this context, the question has been raised as to whether cytosine methylation affects the binding of aflatoxin B_1. Ross *et al*. (1999) examined the effect of CpG methylation on binding of aflatoxin B_1 8,9-epoxide to an 11-mer oligodeoxynucleotide containing the sequence of codons 248 and 249 of the *TP53* gene (see Section 4.4.1(*b*)). Binding to methylated or unmethylated fragments of the human *HPRT* gene was also investigated. In neither instance did cytosine methylation affect aflatoxin B_1 binding to guanines within the sequence. In contrast, Chen *et al*. (1998) reported strongly enhanced binding of aflatoxin B_1 to methylated compared with non-methylated CpG sites in *TP53* mutational hotspots, including codon 248, using an UvrABC incision method. The

difference between the two studies could reflect the different methods, sequence contexts of the target bases or the methods used to detect DNA adducts (Ross *et al*., 1999).

4.5 Mechanistic considerations

4.5.1 *Specificity of 249^{ser} mutation in the TP53 gene*

A number of experimental approaches have been used to examine the plausibility of a causal link between aflatoxin-induced DNA damage and the common AGG to AGT transversion mutation at codon 249 of the *TP53* gene in human HCC from areas where aflatoxin exposure is high. Analysis of *TP53* mutations induced by aflatoxin B_1 reveals that G to T transversion is the most common base substitution (see Section 4.4.1(*b*)). A number of studies have examined the sequence specificity of the induction of either DNA damage or mutations in the *TP53* gene (Puisieux *et al*., 1991; Aguilar *et al*., 1993; Denissenko *et al*., 1998, 1999). In these experimental systems, aflatoxin B_1 induces both damage and mutations at the third nucleotide of codon 249, with some evidence of preferential targeting of this latter site in comparison to the adjacent guanine (second nucleotide of codon 249) or guanines in surrounding codons. However, the degree of targeting to codon 249 does not appear to be sufficient by itself to explain the mutational specificity observed in human HCC. Aflatoxin B_1–DNA adducts at other sites within the *TP53* gene would also induce G to T transversions, with alterations of amino acids and associated changes in p53 protein function, but these mutations are far rarer than the codon 249^{ser} mutation in HCC from areas where aflatoxin exposure is high.

The possible effect of the codon 249^{ser} mutation on p53 protein function and any selective growth advantage conferred on hepatocytes carrying this mutation is also of importance. Overall, the codon 249^{ser} mutation in *TP53* appears insufficient to immortalize human hepatocyte cells in culture, but it does confer a growth advantage to previously immortalized cells (Ponchel *et al*., 1994; Forrester *et al*., 1995; Schleger *et al*., 1999). These cell culture studies are consistent with a selective growth advantage resulting from the codon 249^{ser} mutation, but do not fully explain the high prevalence of this mutation in the *TP53* gene in human HCC. However, in-vitro studies generally do not address the role of the mutation in the intact tissue and the studies mentioned above did not assess the role of co-infection with HBV and its possible influence on both the induction and clonal selection of *TP53* codon 249^{ser} mutations. Sohn *et al*. (2000) transfected human liver epithelial cells with the *HBx* gene — encoding the HBVx protein — and these cells were more sensitive to the cytotoxic action of aflatoxin B_1 8,9-epoxide and to induction of apoptosis and mutations at codon 249, possibly as a result of altered excision repair of the aflatoxin B_1–DNA adduct (Hussain & Harris, 2000).

4.5.2 *Modulation of the effects of aflatoxin with chemopreventive agents*

The understanding of human metabolism of aflatoxin B_1 (see Section 4.1) and the extensive literature on chemoprevention of aflatoxin B_1-induced carcinogenesis in experimental animals have provided a rationale for chemoprevention studies in human populations (Kensler *et al*., 1999). Notably, agents that induce hepatic GST and aflatoxin aldehyde reductase (AFAR) in rats give rise to decreased aflatoxin–DNA and –protein adduct formation and inhibition of aflatoxin-associated carcinogenicity (Roebuck *et al*., 1991; Groopman *et al*., 1992; Judah *et al*., 1993; Kensler *et al*., 1997; Groopman & Kensler, 1999). Consequently, a similar modulation of the balance between aflatoxin activation and detoxification in humans has been sought and two chemopreventive agents, oltipraz and chlorophyllin, have been evaluated in clinical trials in China.

The protective action of oltipraz is based on inhibition of the enzyme CYP1A2, resulting in reduced formation of the aflatoxin B_1 8,9-epoxide and aflatoxin M_1, and induction of GST enzymes, resulting in increased excretion of the 8,9-epoxide glutathione conjugate as a mercapturic acid (Morel *et al*., 1993; Langouët *et al*., 1995). In Chinese subjects exposed to aflatoxin through consumption of their regular diet, concurrent dietary intake of oltipraz was shown to modulate aflatoxin metabolism by increasing excretion of the mercapturic acid and decreasing urinary aflatoxin M_1 concentrations and blood aflatoxin–albumin levels (Jacobson *et al*., 1997; Kensler *et al*., 1998; Wang *et al*., 1999a).

Chlorophyllin is an anti-mutagen in genotoxicity assays *in vitro* and *in vivo* (Dashwood *et al*., 1998). Mechanistic studies of aflatoxin B_1-induced hepatocarcinogenesis in rainbow trout have revealed that chlorophyllin acts as an 'interceptor molecule' through the formation of tight molecular complexes with aflatoxin B_1 (Breinholt *et al*., 1995). Consequently, it may diminish the bioavailability of aflatoxin B_1, leading to reduced DNA-adduct formation and tumour development (Breinholt *et al*., 1999). Chlorophyllin has also been evaluated in a chemoprevention trial in China and consumption of 100 mg of this compound at each meal during four months led to an overall reduction of 55% ($p = 0.036$) in median urinary concentrations of AFB_1–*N*7-Gua compared with placebo controls (Egner *et al*., 2001).

The above clinical trials confirm that aflatoxin metabolism occurring in people exposed to the toxin through the diet is consistent with the metabolic pathways deduced from *in vitro* and animal model studies. These trials also provide proof of principle that aflatoxin metabolism can be modulated *in vivo* to reduce genotoxic damage; this provides a basis for prevention strategies through dietary modulation.

4.5.3 *Interactions of hepatitis B virus and aflatoxins*

In countries with a high incidence of HCC, endemic infection with HBV is often associated with exposure to aflatoxins. Prospective cohort studies from Asia have observed a multiplicative increase in risk for HCC in individuals chronically infected with HBV and exposed to dietary aflatoxins (see Section 2). Experimental studies in

HBV-transgenic mice and woodchucks also suggest a synergism between the two risk factors in the induction of HCC (Sell *et al.*, 1991; Bannasch *et al.*, 1995). An understanding of the molecular mechanisms behind this interaction is relevant to public health measures aimed at reducing HCC incidence. These mechanisms are considered briefly below; for more detailed information, the reader is referred to a number of review articles (Harris & Sun, 1986; Wild *et al*., 1993a,b; JECFA, 1998; Sylla *et al*., 1999; Wild & Hall, 1999).

One possible mechanism of interaction between the virus and the chemical carcinogen is HBV infection altering the expression of aflatoxin-metabolizing enzymes. This has been addressed most extensively in HBV-transgenic mouse lineages carrying the gene for HBsAg, where induction of specific CYP isozymes, namely 1A and 2A5, is observed in association with expression of the *HBsAg* transgene (Chemin *et al.*, 1996; Kirby *et al.*, 1994a), but only in lineages in which transgene expression was associated with induction of liver injury (Chomarat *et al*., 1998; Chemin *et al*., 1999). Similar induction of CYP enzymes is observed with liver injury associated with bacterial and parasitic infections (Kirby *et al.*, 1994b; Chomarat *et al.*, 1997), suggesting a general mechanism involving liver injury *per se* rather than a specific effect of HBV on enzyme expression. The modifying effects of HBV-related transgene expression are not limited to CYP enzymes but also include effects on GST enzymes (Chemin *et al*., 1999). Kirby *et al.* (1996a) showed increased CYP2A6 and CYP3A4 activity in human liver in relation to hepatitis infection. Human liver specimens with evidence of HBV infection had significantly lower total GST activity than non-infected livers (Zhou *et al.*, 1997). In HBV-transfected HepG2 human hepatoma cells, expression of GST α-class enzymes was significantly decreased (Jaitovitch-Groisman *et al*., 2000).

Some studies have examined aflatoxin metabolism in HBV-infected individuals exposed to the toxin through the diet. Cortisol metabolism was measured as a marker of CYP3A4 activity in relation to aflatoxin–albumin adducts in 357 Gambian adults. No association was observed between CYP3A4 activity and either HBV infection status or adduct levels. The level of aflatoxin–albumin adduct was not related to the HBV status of the individual, including HBV DNA and HBe antigen, which are markers of active viral replication. In addition, there was no correlation between adducts and serum transaminases, markers of liver injury (Wild *et al*., 2000). In a study of nine adult Gambian HBV carriers and 11 non-carriers with measured dietary intakes of aflatoxin, no differences in levels of urinary aflatoxin–DNA adducts were observed between the two groups (Groopman *et al*., 1992). These data suggest that the aflatoxin–albumin and urinary aflatoxin–DNA adducts are not influenced by HBV infection in adults. In contrast, in HBV-infected Gambian children there was a higher level of aflatoxin–albumin adducts than in non-infected children, an observation consistent with altered aflatoxin metabolism (Allen *et al.*, 1992; Turner *et al*., 2000). Similar observations of higher aflatoxin–albumin adduct levels in HBsAg carriers have been reported in a study of 200 Chinese adolescents (Chen *et al*., 2001).

Thus overall, there is potential for HBV infection to modulate aflatoxin metabolism but the effects are likely to be complex, involving the possibility of both altered activation and detoxification.

An alternative mechanism of interaction between HBV and aflatoxin is that carcinogen exposure may modulate the course of viral infection and replication. In ducklings infected with duck hepatitis virus and treated with aflatoxin B_1, there was a significant increase in markers of viral replication in liver and serum (Barraud *et al.*, 1999, 2000), supporting the hypothesis that aflatoxins can enhance hepadnaviral gene expression. Human hepatoma HepG2 cells transfected with HBV and treated with aflatoxin B_1 also showed an increase in the concentration of transcription factors that may influence HBV expression (Banerjee *et al.*, 2000).

A further hypothesis for the probable interaction of the two risk factors is that the DNA adducts formed by aflatoxin are more likely to be fixed as mutations in the presence of the increased cell proliferation induced by chronic HBV infection.

In summary, plausible mechanisms of interaction between aflatoxins and HBV exist, but to date no conclusion can be drawn as to the most relevant mechanism in terms of the synergistic effects observed in epidemiological studies.

5. Summary of Data Reported

5.1 Exposure data

Aflatoxins are a family of fungal toxins produced mainly by two *Aspergillus* species which are especially abundant in areas of the world with hot, humid climates. *Aspergillus flavus*, which is ubiquitous, produces B aflatoxins. *A. parasiticus*, which produces both B and G aflatoxins, has more limited distribution. Major crops in which aflatoxins are produced are peanuts, maize and cottonseed, crops with which *A. flavus* has a close association. Human exposure to aflatoxins at levels of nanograms to micrograms per day occurs mainly through consumption of maize and peanuts, which are dietary staples in some tropical countries. Maize is also frequently contaminated with fumonisins. Aflatoxin M_1 is a metabolite of aflatoxin B_1 in humans and animals. Human exposure to aflatoxin M_1 at levels of nanograms per day occurs mainly through consumption of aflatoxin-contaminated milk, including mothers' milk. Measurement of biomarkers is being used increasingly to confirm and quantify exposure to aflatoxins.

5.2 Human carcinogenicity data

Studies evaluated in Volume 56 of the *IARC Monographs* led to the classification of naturally occurring aflatoxins as *carcinogenic to humans (Group 1)*. Recent studies have incorporated improvements in study design, study size and accuracy of measurement of markers of exposure to aflatoxin and hepatitis viruses.

In a large cohort study in Shanghai, China, risk for hepatocellular carcinoma was elevated among people with aflatoxin metabolites in urine, after adjustment for cigarette smoking and hepatitis B surface antigen positivity. No association was observed between dietary aflatoxin levels, as ascertained by a diet frequency questionnaire, and risk for hepatocellular carcinoma.

There were four reports from cohort studies in Taiwan, China, although three of them partly overlapped. Selected subjects in the three overlapping studies were enrolled, were interviewed, had biological specimens taken, and were followed up intensively for liver cancer. In nested case–control studies, including some prevalent cases, subjects with exposure to aflatoxin, as assessed by biomarker measurements, had elevated risks for liver cancer, after adjustment for hepatitis B surface antigen positivity. The effect due to aflatoxin exposure was especially high among those who were positive for hepatitis B surface antigen, but there were few liver cancer cases negative for hepatitis B surface antigen. The other Taiwan study was carried out in a large cohort of chronic carriers of hepatitis B virus. These subjects were interviewed at baseline, had biological specimens taken, and were followed up intensively for liver cancer. Several aflatoxin metabolites and albumin adducts were measured in a nested case–control series. Subjects with quantified levels of most of the biomarkers of exposure to aflatoxin showed elevated risk for liver cancer.

In two studies in Qidong, China, cohorts of hepatitis B carriers were tested for biomarkers of aflatoxin and followed up for liver cancer. In both studies, subjects with aflatoxin biomarkers had excess risks for liver cancer.

In a Sudanese case–control study of liver cancer, a relationship was found between reported ingestion of peanut butter and liver cancer in a region with high aflatoxin contamination of peanuts, but no such relationship in a region with low contamination of peanuts.

In a hybrid ecological cross-sectional study in Taiwan, China, a number of subjects were selected from eight regions; for each subject several biomarkers of aflatoxin and hepatitis B viral infection were assessed in relation to the liver cancer rates in the region of residence. There were correlations between aflatoxin metabolites and liver cancer rates after adjustment for hepatitis B status.

The overall body of evidence supports a role of aflatoxins in liver cancer etiology, notably among subjects who are carriers of hepatitis B surface antigen. Nevertheless, the interpretation of human studies is hampered by the difficulties in properly assessing an individual's lifetime exposure to aflatoxins and the difficulties in disentangling the effects of aflatoxins from those of hepatitis infections. Novel biomarkers, some still under development and validation, should bring greater clarity to the issue.

5.3 Animal carcinogenicity data

Extensive experimental studies on the carcinogenicity of aflatoxins led to a previous *IARC Monographs* evaluation of the evidence as follows: *sufficient evidence* for carcino-

genicity of naturally occurring mixtures of aflatoxins and of aflatoxins B_1, G_1 and M_1, *limited evidence* for aflatoxin B_2 and *inadequate evidence* for aflatoxin G_2. The principal tumours induced were liver tumours.

Carcinogenicity studies in experimental animals since 1993 were limited to a few experiments in rats, trout, mice, tree shrews and woodchucks. Under certain conditions, including increased pressure, decontamination of feed containing aflatoxins by ammoniation almost completely eliminated the induction of hepatic tumours in rats. Studies in trout showed that ammoniation of aflatoxin-contaminated maize significantly reduced the incidence of liver tumours. In trout fed non-fat dried milk from cows fed ammoniated or non-ammoniated aflatoxin-contaminated whole cottonseed, ammoniation almost eliminated the liver tumour response. Less hepatic tumours were induced in trout after exposure to aflatoxin M_1 than with aflatoxin B_1. One aflatoxin metabolite, aflatoxicol, elicited a slightly higher hepatic tumour response in fry and fish embryos than aflatoxin B_1.

A study in transgenic mice overexpressing transforming growth factor β showed no increased susceptibility to induction of hepatocellular adenomas and carcinomas after intraperitoneal administration of aflatoxin B_1. In another study, induction of hepatocellular tumours by aflatoxin B_1 was significantly enhanced in transgenic mice heterozygous for the *TP53* gene and expressing hepatitis B surface antigen. The tumour response for aflatoxin B_1 was reduced in the absence of either one of these risk factors. The presence of the *TP53* 246^{ser} mutant not only enhanced the synergistic effect of hepatitis B virus and aflatoxin B_1 but also increased tumorigenesis due to aflatoxin B_1 in the absence of hepatitis B virus.

In tree shrews, the incidence of hepatocellular carcinomas was significantly increased and the time of occurrence was shortened in animals treated with aflatoxin B_1 and infected with (human) hepatitis B virus compared with aflatoxin B_1-treated animals. Woodchucks infected with woodchuck hepatitis virus were more sensitive to the carcinogenic effects of aflatoxin B_1 than uninfected woodchucks. The combined woodchuck hepatitis virus/aflatoxin B_1 treatment not only reduced the time of appearance but also resulted in a higher incidence of liver tumours.

In conclusion, recent studies continue to confirm the carcinogenicity of aflatoxins in experimental animals.

5.4 Other relevant data

Metabolism of aflatoxin B_1 in humans has been well characterized, with activation to aflatoxin B_1 8,9-*exo*-epoxide resulting in DNA adduct formation. CYP1A2, 3A4, 3A5, 3A7 and GSTM1 enzymes among others mediate metabolism in humans. The expression of these enzymes can be modulated with chemopreventive agents, resulting in inhibition of DNA-adduct formation and hepatocarcinogenesis in rats. Oltipraz is a chemopreventive agent that increases glutathione conjugation and inhibits some cytochrome P450 enzymes. Results from clinical trials in China using oltipraz are consistent with

experimental data in showing that, following dietary exposure to aflatoxins, modulation of the metabolism of aflatoxins can lead to reduced levels of DNA adducts.

Aflatoxin B_1 is immunosuppressive in animals, with particularly strong effects on cell-mediated immunity. Exposure to aflatoxin results in increased susceptibility to bacterial and parasitic infections. Human monocytes treated with aflatoxin B_1 had impaired phagocytic and microbicidal activity and decreases in specific cytokine secretion. Studies have linked human exposure to aflatoxins to increased prevalence of infection.

Aflatoxins cross the human placenta. Aflatoxin exposure has been associated with growth impairment in young children. Malformations and reduced fetal weight have been seen after mice were treated intraperitoneally with high doses of aflatoxin. In rats, decreased pup weight and behavioural changes have been found at low doses. Effects suggesting impairment of fertility have been reported in female and male rats and in male rabbits.

Aflatoxin B_1 is genotoxic in prokaryotic and eukaryotic systems *in vitro*, including human cells, and *in vivo* in humans and in a variety of animal species. It forms DNA and albumin adducts and induces gene mutations and chromosomal alterations including micronuclei, sister chromatid exchange and mitotic recombination.

In geographical correlation studies, exposure to aflatoxin is associated with a specific G to T transversion in codon 249 of the *TP53* gene in human hepatocellular carcinoma. This alteration is consistent with the formation of the major aflatoxin B_1–*N*7-guanine adduct and the observation that G to T mutations are predominant in cell and animal model systems. The high prevalence of the codon 249 mutation in human hepatocellular carcinoma, however, is not fully explained in experimental studies either by the sequence-specific binding and mutation induced by aflatoxin B_1 or by altered function of the p53 protein in studies of hepatocyte growth and transformation.

Current knowledge of the molecular mechanisms contributes to the understanding of the nature of the interaction between hepatitis B virus and aflatoxins in determining risk for hepatocellular carcinoma. Infection with hepatitis B virus may increase aflatoxin metabolism; in hepatitis B virus-transgenic mice, liver injury is associated with increased expression of cytochrome P450 (CYP) enzymes. Glutathione *S*-transferase activity is also reduced in human liver in the presence of hepatitis B virus infection. Other molecular mechanisms are, however, also likely to be relevant to aflatoxin-induced carcinogenesis.

On the basis of the data described above, the existing Group 1 evaluation of naturally occurring aflatoxins was reaffirmed.

5.5 Further research needs

Some research areas are identified here for the purpose of assisting in any future update by an IARC Monographs Working Group. It is not implied that these areas listed

override the importance of other research areas or needs, nor should this be construed as endorsement for any specific studies planned or in progress.

- Development and use of molecular dosimetry and/or biomarkers to identify high-risk groups, e.g., in view of children's susceptibility
- Further study of interaction between aflatoxin B_1 and hepatitis B virus and the role of both factors in hepatocarcinogenesis
- Use of biomarker measurements in assessing the association between aflatoxins and hepatocellular carcinoma in epidemiological studies
- Pharmacokinetic studies of ingested aflatoxins in humans (with and without liver disease)
- New epidemiological studies of, for example, liver cancer among populations vaccinated against hepatitis B, populations with exposure to aflatoxins and (limited) exposure to hepatitis B virus, as in Latin America, and joint effects of aflatoxins and hepatitis C virus on liver cancer.

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ANNEX. AFLATOXINS IN FOODS AND FEEDS: FUNGAL SOURCES, FORMATION AND STRATEGIES FOR REDUCTION

1. Major Mycotoxins and Crops

Five types of mycotoxins of agricultural importance occur in staple crops (Miller, 1995): aflatoxins, fumonisins, ochratoxin A, specific trichothecenes (deoxynivalenol and nivalenol) and zearalenone. These mycotoxins can cause various forms of poisoning in animals and in humans, and some are carcinogenic (Table 1). Aflatoxins are produced by *Aspergillus* species in nuts and oilseeds, particularly maize, peanuts (groundnuts) and cottonseed, especially in tropical and subtropical climates. Fumonisins are produced by *Fusarium verticillioides* (formerly known as *F. moniliforme*) and the closely related *F. proliferatum*, in maize and sorghum. Ochratoxin A occurs in cereals as a result of growth of *Penicillium verrucosum* and in other crops, especially grape products (grape juice, wines and dried vine fruit), coffee and long-stored commodities as a result of growth of several *Aspergillus* species. Deoxynivalenol, nivalenol and zearalenone are formed as a result of growth of *F. graminearum* and *F. culmorum* in maize, wheat, barley and other small grains (JECFA, 2001). Ergot, the toxic product of the fungus *Claviceps purpurea* which grows on rye and to a lesser extent on other grains, was historically a significant source of epidemic poisoning in Europe, but due to effective inspection of grain it is rarely a public health problem today in Europe and North America.

Some crops are infected by only one toxigenic fungus: aflatoxins in peanuts and cottonseed, and ochratoxin A in susceptible crops are usually found by themselves. In small grains, trichothecenes and zearalenone usually occur together as the result of infection by one or more *Fusarium* species. Of greater importance, fumonisins and aflatoxins, and to a lesser extent trichothecenes and zearalenone, frequently occur simultaneously in maize. Any conclusions regarding reduction strategies should take this into account.

This Annex deals only with aflatoxins, but many of the points made have relevance to the other toxins.

Table 1. IARC evaluations

Previous evaluation[a]				**This volume**			
Agent	Degree of evidence of carcinogenicity		Overall evaluation of carcinogenicity to humans	Agent	Degree of evidence of carcinogenicity		Overall evaluation of carcinogenicity to humans
	Human	Animal			Human	Animal	
Aflatoxins, naturally occurring mixtures of	S	S	1	Naturally occurring aflatoxins			1 (reaffirmed)
Aflatoxin B_1	S	S					
Aflatoxin B_2		L					
Aflatoxin G_1		S					
Aflatoxin G_2		I					
Aflatoxin M_1	I	S	2B				
Toxins derived from *Fusarium moniliforme* (now called *F. verticillioides*)	I	S	2B				
Fumonisin B_1		L		Fumonisin B_1	I	S	2B
Fumonisin B_2		I					
Fusarin C		L					
Ochratoxin A	I	S	2B				
Trichothecenes							
Toxins derived from *Fusarium graminearum* and *F. culmorum*	I		3				
Zearalenone		L					
Deoxynivalenol		I					
Nivalenol		I					

S, sufficient evidence of carcinogenicity; L, limited evidence of carcinogenicity; I, inadequate evidence of carcinogenicity; group 1, carcinogenic to humans; group 2B, possibly carcinogenic to humans; group 3, not classifiable as to its carcinogenicity to humans

[a] IARC (1993) *IARC Monographs on the Evaluation of Carcinogenic Risks to Humans*, Vol. 56, *Some Naturally Occurring Substances: Food Items and Constituents, Heterocyclic Aromatic Amines and Mycotoxins*, Lyon, IARC*Press*

2. Aflatoxins

2.1 Introduction

Aflatoxins frequently contaminate certain types of foods and feeds in warm and tropical regions. Limiting the formation of aflatoxins in such commodities cannot usually be achieved by any single technique. To be effective, approaches to this problem require an overall strategy, which involves a knowledge of crops likely to be affected, the time when infection by the fungi is likely to occur, and careful management of crops both before and after harvest. Once aflatoxins have been formed, reduction again relies on a range of strategies, which if well managed can result in the removal of the major part of aflatoxin contamination. Contaminated crops can be used for animal feed after chemical treatment. This Annex provides an overview of the crops and conditions that favour aflatoxin contamination, and the various management strategies available to limit formation or reduce levels of aflatoxins in commodities.

2.2 Fungi producing aflatoxins

Aflatoxins in food and feed crops are almost entirely produced by the common fungi *Aspergillus flavus* and the closely related species *A. parasiticus*. *A. flavus* produces only B aflatoxins while *A. parasiticus* produces both B and G aflatoxins (Schroeder & Boller, 1973; Dorner *et al.*, 1984; Klich & Pitt, 1988; Pitt, 1993). Several other *Aspergillus* species are now known to produce aflatoxins but they are of little practical importance in foods.

2.3 Occurrence of toxigenic species in foods

2.3.1 *Aspergillus flavus*

A. flavus is the most widely reported food-borne fungus outside north-temperate areas. It is especially abundant in the tropics: *A. flavus* was isolated from 97% of nearly 500 peanut samples examined from south-east Asian sources over the years 1989–91, with an average infection rate of more than 40% of all surface-disinfected kernels examined from Thailand and the Philippines, and more than 60% of those from Indonesia. For maize, the figures were 89% of 380 samples, at an average of 38% of all grains infected from Indonesia and the Philippines, and 17% of those from Thailand (Pitt & Hocking, 1997).

Levels in food commodities from more temperate climates, such as Australia or the USA, are much lower. *A. flavus* was present in only 1.2% of surface-disinfected maize

kernels and in less than 0.1% of all oats and wheat kernels examined from a large number of samples in the USA (Sauer *et al.*, 1984).

The major food and feed commodities where *A. flavus* is found are peanuts (McDonald, 1970; Pitt *et al.*, 1993, 1998), maize (Diener *et al.*, 1983; Pitt *et al.*, 1993, 1998) and cottonseed (Simpson & Batra, 1984). Spices of many kinds frequently contain *A. flavus* (ICMSF, 1998). From time to time, *A. flavus* occurs in most types of tree nuts, including pistachios, pecans, hazelnuts and walnuts, copra and kola nuts (Pitt & Hocking, 1997). Aflatoxins are sometimes produced in these commodities (Pohland & Wood, 1987). Low levels of *A. flavus* in small grain cereals and pulses, and many other kinds of foods, e.g. soybean, have been reported, but the possibility of significant aflatoxin accumulation is much lower (Pitt *et al.*, 1994; Pitt & Hocking, 1997; Pitt *et al.*, 1998).

2.3.2 *Aspergillus parasiticus*

A. parasiticus seems to be less widely distributed than *A. flavus*. During a major study, more than 30 000 *A. flavus* cultures from south-east Asian foods were isolated and identified, but not more than 20 isolates of *A. parasiticus* were found. Although *A. parasiticus* is certainly widely distributed in soils and foodstuffs in the USA, Latin America, South Africa, India and Australia, it is essentially unknown in south-east Asia. Like *A. flavus*, it is a tropical and subtropical species, less prevalent in warm temperate zones, and rare in the cool temperate regions of the world. The most important food source is peanuts, in which *A. parasiticus* is endemic. Other types of nuts may be infected, including hazelnuts and walnuts, pistachios and pecans. *A. parasiticus* is much less common than *A. flavus* on grains, and perhaps does not invade maize at all. A variety of other minor sources have been reported (Pitt & Hocking, 1997).

2.4 Formation of aflatoxins in foods

A fundamental distinction must be made between aflatoxins formed in crops before or immediately after harvest, and those occurring in stored commodities or food products. In subtropical and tropical areas, certain crop plants, notably peanuts, maize and cottonseed are associated with *A. flavus*, or in the case of peanuts, also for *A. parasiticus*, so that invasion of plants and developing seeds or nuts may occur before harvest. This is the cause of the frequent occurrence of high levels of aflatoxins in these crops, and is the reason for the difficulties still being experienced in eliminating aflatoxins from these commodities. In contrast, *A. flavus* is less common in other plants, seeds or nuts before harvest. In consequence, aflatoxins are not normally a problem with other crops at harvest and their elimination relies on preventing post-harvest contamination, by rapid drying and good storage practice (Pitt, 1989; Chatterjee *et al.*, 1990; Miller, 1995). Therefore, if infection of peanuts and maize by *A. flavus* could be controlled before harvest, excessive aflatoxin production would not normally occur in storage, even under somewhat unsatisfactory conditions. In temperate maize production, *A. flavus* conta-

mination is associated mainly with insect damage during drought conditions (Miller, 1995).

2.5 Formation of aflatoxins in susceptible crops

2.5.1 *Peanuts*

Peanuts are susceptible to infection by both *A. flavus* and *A. parasiticus* (Hesseltine *et al.*, 1970; Diener *et al.*, 1987; Pitt & Hocking, 1997). The primary source of these fungi is soil, where high numbers may build up because some peanuts are not harvested, but remain in the ground and act as a nutrient source (Griffin & Garren, 1976a). Uncultivated soils contain very low amounts of *A. flavus*, but soils in peanut fields usually contain 100–5000 propagules (spores) per gram (Pitt, 1989). Under drought stress conditions, this number may rise to 10^4 or 10^5/g (Horn *et al.*, 1995). Large numbers of *A. flavus* spores are also airborne over susceptible crops (Holtmeyer & Wallin, 1981).

Direct entry to developing peanuts through the shell by *A. flavus* in the soil appears to be the main route of nut infection (Diener *et al.*, 1987). Infection can also occur through the pegs and flowers (Wells & Kreutzer, 1972; Griffin & Garren, 1976b; Pitt, 1989). *A. flavus* sometimes grows within peanut plants themselves. Growth in plant tissue is not pathogenic, but commensal: neither the seed pod (Lindsey, 1970) nor the plant (Pitt, 1989; Pitt *et al.*, 1991) shows any visible sign of colonization by the fungus.

A variety of factors influence invasion of developing peanuts by *A. flavus*. Infection occurs before harvest only if substantial numbers of spores or other propagules (thousands per gram of soil) exist in the soil. Other important factors are drought stress (Sanders *et al.*, 1981) and soil temperatures around 30 °C (Blankenship *et al.*, 1984; Sanders *et al*., 1984; Cole *et al.*, 1985; Cole, 1989; Dorner *et al.*, 1989) during the last 30–50 days before harvest (Sanders *et al.*, 1985) .

2.5.2 *Maize*

So far as is known, maize is infected only by *A. flavus* (Lillehoj *et al.*, 1980; Angle *et al.*, 1982; Horn *et al.*, 1995). In temperate areas, the most important route for entry of *A. flavus* to maize is through insect damage (Lillehoj *et al.*, 1982; Bilgrami *et al.*, 1992; Miller, 1995). Invasion via the silks (the styles of the female maize flower) is also possible (Marsh & Payne, 1984; Diener *et al.*, 1987). High-temperature (32–38 °C) stress increases infection (Jones *et al.*, 1980). The critical time for high temperatures to favour infection is between 16 and 24 days after inoculation at silking (Payne, 1983). The time of infection is also important: inoculation two to three weeks after silk emergence produced much higher rates of infection than inoculation one or five weeks after silking (Jones *et al.*, 1980).

2.5.3 *Cottonseed*

A. flavus is also a commensal in the cotton plant (Klich *et al*., 1984). Infection occurs through the nectaries (natural openings in the cotton stem), which are important in pollination (Klich & Chmielewski, 1985), or through cotyledonary leaf scars (Klich *et al*., 1984). Upward movement occurs in the stem towards the boll, but not downwards from boll to stem (Klich *et al.*, 1986). Insect damage is also a potential cause of infection (Lee *et al.*, 1987), but insects are often well controlled in cotton crops. As in peanuts and maize, temperature appears to be a major environmental factor in pre-harvest infection of cottonseed (Marsh *et al.*, 1973; Simpson & Batra, 1984). Daily minimum temperatures above 24 °C, in combination with precipitation exceeding 2–3 cm, appear to lead to extensive aflatoxin formation (Diener *et al.*, 1987).

3. Management of Aflatoxin Contamination

A variety of approaches exist to limit aflatoxin production in crops. Limiting aflatoxins in crops before and immediately after harvest involves strategies aimed at reducing drought stress, by irrigation and weed control, by control of insect damage and by the use of fungicides. Improvements in harvesting procedures, better drying, and sorting of defective grains or nuts are all beneficial. The principles of safe storage are well known, and cannot be overemphasized. Physical methods to reduce aflatoxins in crops are widely practised, especially in developed countries. The technology involved in these approaches to limiting and reducing aflatoxins are relatively simple and inexpensive, and can be practised even by small-scale farmers. For heavily contaminated commodities, the use of chemical treatments can effectively reduce aflatoxins, but then use of the resulting materials is limited to animal feed. These procedures, systems and approaches are outlined below.

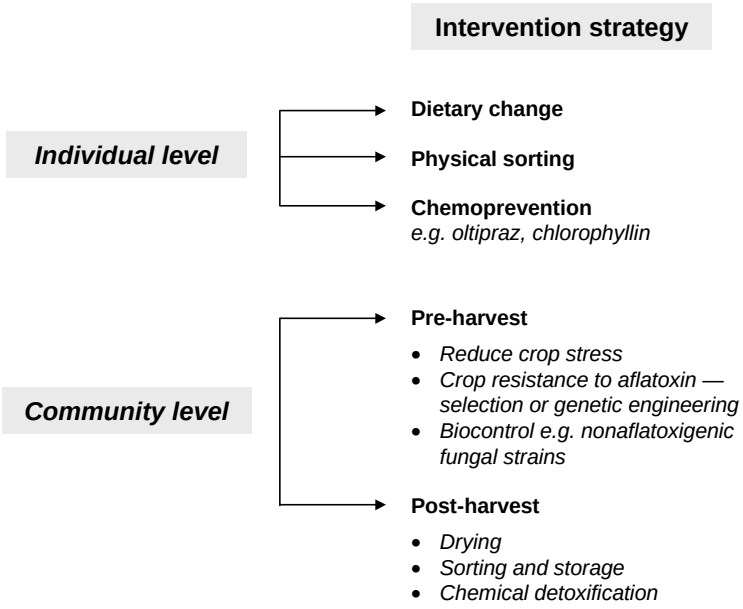
3.1 Intervention strategies

Interventions to reduce aflatoxin-related exposures can be considered in terms of those which are applicable at the individual level or those applicable at the community level (Figure 1).

3.1.1 *Individual level*

Dietary changes to avoid foods contaminated with aflatoxin are rarely an option in countries of high exposure where staple foods are contaminated (e.g. maize or peanuts). However, efforts to improve crop and dietary diversity must be made. Sorting procedures can be of value, but require education at the consumer level. Chemoprevention aims to diminish the toxicological effects of aflatoxins once exposure has occurred. Clinical

Figure 1. Intervention strategies



trials of two chemopreventive agents, oltipraz and chlorophyllin, have demonstrated that aflatoxin metabolism can be modified *in vivo* and the levels of aflatoxin bound to DNA and proteins can be reduced as a result. This approach may be valuable in individuals at particularly high risk of exposure. Work on these chemical agents also provides a valuable scientific basis for the exploration of dietary constituents consumed by populations exposed to aflatoxins, which may modify the toxicity of the aflatoxins in a similar way.

3.1.2 *Community level*

(*a*) *Limiting aflatoxin formation in susceptible crops before and after harvest*

In peanuts, it appears likely that infection by *A. flavus* while nuts are still in the ground is a prerequisite for high levels of aflatoxins to be formed after harvest (Pitt, 1989). In the absence of high pre-harvest infection levels, and with rapid and effective drying, peanuts can be produced free of any appreciable level of aflatoxin. The major causes of pre-harvest infection are high numbers of propagules in the soil, and drought stress during the days leading up to harvest (see Section 2.5.1).

Partial control of propagule numbers can be achieved by crop rotation: in particular, numbers of *A. flavus* in soil decrease under small grain cultivation or pasture. Irrigation, which eliminates drought stress, is regarded as the most effective method for reducing

aflatoxin formation in peanuts (Pettit *et al.*, 1971; Cole *et al.*, 1982). However, peanuts throughout the world are recognized as a drought-resistant crop and are mostly grown under dry culture, with irrigation reserved for more moisture-sensitive crops such as rice or vegetables. In many areas where peanuts are grown, irrigation is not an option. Under these circumstances, reduction in drought stress by good agricultural practices can be a beneficial approach. For example, weed control and wider spacing between peanut rows can both assist in reducing drought stress (Rachaputi, 1999). Rapid drying of peanuts using mechanical dryers as soon as possible after pulling has a major effect in reducing the levels of aflatoxins in peanuts.

For maize also, irrigation and improved farm management practices have a beneficial effect on aflatoxin formation (Payne *et al.*, 1986). Resistant breeding stocks have been identified (Widstrom *et al.*, 1987; Campbell & White, 1995) and resistant maize genotypes, dependent on kernel pericarp wax (Guo *et al.*, 1995; Brown *et al.*, 1999) or kernel proteins (Huang *et al.*, 1997) have been developed recently. As maize is usually dried in the field, rapid drying techniques are not commonly practised, but should be in tropical countries.

Cottonseed is a by-product of cotton production, so field drying is normal. Breeding of cotton without nectaries has been proposed as one means of limiting *A. flavus* access to cotton bolls (Klich & Chmielewski, 1985).

Genetic engineering may offer novel ways of limiting pre-harvest contamination by mycotoxins, provided attention is paid to questions of importance specifically to developing countries (Wambugu, 1999). Genetic approaches to aflatoxin control include engineering of genes in *Aspergillus* species to influence the ability of the fungus to colonize the host plant. An alternative approach is to select or engineer varieties of cereal grains and oilseeds resistant to fungal infection or aflatoxin biosynthesis by the fungus once infection occurs.

(*b*) *Control of aflatoxin formation in other crops before and after harvest*

Entry of *A. flavus* into pistachio nuts depends on the time of splitting of hulls. Nuts in which hull splitting occurs early are much more susceptible to *A. flavus* invasion on the tree (Doster & Michailides, 1995). It is known that some cultivars are more prone to early splitting than others, and this is especially important where nuts are harvested from the ground, after contact with the soil.

In tree nut crops, various techniques, including timing of harvesting, are used to keep aflatoxin formation to a minimum.

Figs are sometimes infected by *A. flavus*, both because of their unique structure developed for insect fertilization and also because figs are harvested from the ground in some countries. Immature figs are not colonized by *A. flavus*, but once they are ripe infection occurs readily and fungal growth continues during drying (Buchanan *et al.*, 1975; Le Bars, 1990). The proportion of figs infected is only about 1% (Steiner *et al.*, 1988).

(*c*) *Control of aflatoxins in dried food commodities by physical means*

A range of physical factors control fungal growth: temperature, water activity (a_w), pH, gas atmospheres and oxygen concentration (Pitt & Hocking, 1997) together with the use of insecticides or preservatives in some cases. The pH of any commodity cannot be altered (Wheeler *et al.*, 1991), while temperature is usually not controllable in bulk storage. Gas atmospheres are increasingly used in developed countries to limit insect growth, and, if well maintained, can also limit fungal growth (Hocking, 1990). Storage of grain in a phosphine atmosphere used to control insects at a water activity of 0.80 or 0.86 reduced growth of *Aspergillus flavus*, but had little effect on the survival of spores (Hocking & Banks, 1991). In practice, in most storage systems, a_w is the principal variable that can be modified for preserving commodities.

The basic advice for handling any grain, nut, other bulk food commodity or feed after harvesting is to dry it rapidly and completely and to keep it dry. Food commodities are perishable, and must be kept free of insect infestations or water ingress or heating and cooling gradients which will cause migration of moisture. Full description of the methods for successful storage is beyond the scope of this document. Many good texts on grain storage exist: those by Champ and Highley (1988), Champ *et al.* (1990) and Highley *et al.* (1994) are recommended.

The prime consideration for storage of grains and nuts is to maintain the moisture content below that which permits fungal growth of any sort over a normal storage life, about one year, i.e. at a water activity below 0.65. This corresponds to different moisture contents for different commodities: 8% for peanuts and other nuts, 12% for grains and 22% for raisins, which contain a higher level of soluble carbohydrate (Iglesias & Chirife, 1982).

The limits for growth of *A. flavus* are now reasonably well defined: *A. flavus* is able to grow between 10–12 °C and 43–48 °C, with an optimum near 33 °C (Pitt & Hocking, 1997); the minimum a_w for growth is near 0.82 at 25 °C, 0.81 at 30 °C and 0.80 at 37 °C (Pitt & Miscamble, 1995). Growth can occur over the pH range 2–11 at least, at 25–37 °C, with optimal growth over a broad range from pH 3.4 to 10 (Wheeler *et al.*, 1991). *A. parasiticus* is very similar physiologically to *A. flavus* (Pitt & Hocking, 1997). Data from Pitt and Miscamble (1995) were used to provide a predictive model for *A. flavus* growth in relation to water activity and temperature (Gibson *et al.*, 1994).

Aflatoxin production has been reported to occur at water activity as low as 0.82 but is very slow below about 0.90 and optimal above 0.99, i.e. near the water activity of fresh grains or nuts (ICMSF, 1996; Gqaleni *et al.*, 1997).

It is evident that reduction of water activity of fresh commodities to below 0.80 will positively prevent aflatoxin production. However, it must be kept in mind that holding commodities above a water activity of 0.65 renders them susceptible to the growth of fungi. As such fungi grow, they release water by metabolism and produce heating, both of which in due course lead to conditions conducive to aflatoxin production. To prevent

aflatoxin production in stored commodities, bulk foods or feeds, water activity must be maintained below 0.70 (Pitt & Hocking, 1997).

(*d*) *Reduction of aflatoxins in stored commodities by physical means*

For some crops, notably maize and figs, it is possible to sort grains or fruit using ultraviolet light, under which aflatoxins (and perhaps some other compounds) produce bright greenish yellow fluorescence. This test (qualitative but not quantitative) is best carried out on cracked maize grains (Shotwell *et al*., 1972; Shotwell, 1983), but can be used on whole dried figs (Steiner *et al.*, 1988; Le Bars, 1990). Sorting out contaminated fruit, together with toxin analysis, has been effective in controlling aflatoxins in figs (Sharman *et al.*, 1991). Sieving of contaminated maize has been shown to reduce aflatoxin and the co-occurring fumonisin (Murphy *et al*., 1993).

During wet milling of maize, aflatoxin is segregated primarily in the steep water (40%) and fibre (38%) and germ (6%), with less in the gluten (15%) and starch (1%) (Bennett & Anderson, 1978; Wood, 1982; Njapau *et al*., 1998). In dry milling of maize, artificially contaminated rice and durum wheat, less than 10% of the aflatoxin in the original material remained in the prime products (grits and low-fat flour) (Schroder *et al*., 1968; Scott, 1984).

After peanuts are shelled, several physical procedures such as colour sorting, density flotation, blanching and roasting are routinely used by processors to reduce aflatoxin levels by 99% (Park, 1993a; López-García *et al.*, 1999). The colour sorting process was developed originally to reject commercially unacceptable discoloured nuts, regardless of cause, but as fungal growth is a prime cause of discolouration, the process is also an effective non-destructive means of removing most nuts containing aflatoxins. In crops under severe drought stress, peanuts begin to dry in the ground, and under these conditions luxuriant growth of *A. flavus* can occur, with high aflatoxin production. In this case, blanching to remove skins and roasting to increase discolouration permits effective colour sorting to be carried out. Roasted peanuts must be sold under inert gas atmospheres to suppress development of rancidity (Read, 1989).

Colour sorting of other commodities is not easy. No effective non-chemical testing technique exists for cottonseed or pistachios and, as with other commodities, non-destructive chemical assays are not available.

The extent to which aflatoxins are destroyed during heating is largely dependent on the process used. Less than 25% of the aflatoxin content of a commodity is destroyed by boiling water (Christensen *et al*., 1977; Njapau *et al.*, 1998), extrusion (Cazzaniga *et al*., 2001) and autoclaving (Stoloff *et al*., 1978). However, dry roasting of peanuts can reduce aflatoxin levels by up to 80% (Conway *et al*., 1978; Njapau *et al*., 1998). Heating of peanut oil at 250 °C for 3.5 h reduced aflatoxin by 99% (Peers & Linsell, 1975).

Heating at neutral pH at 125 °C had little effect on fumonisin, usually present as a co-contaminant in maize, but heating above 150 °C causes significant reduction in levels of fumonisin in processed maize products (Dupuy *et al*., 1993; Jackson *et al*., 1996a,b).

3.2 Chemical methods

The alkali process usually practised to produce refined table oil completely removes aflatoxin (ICMSF, 1996).

The use of chemicals to inactivate, bind or remove aflatoxins has been studied extensively. Any such procedure must effectively inactivate or remove the toxin, while maintaining the nutritional and technological properties of the product and without generating toxic reaction products (López-García & Park, 1998). Food safety demonstration studies must be conducted to ensure compliance with regulatory requirements. To date, these chemical methods have been approved only for the reduction of aflatoxins in animal feed commodities. Reacting the toxin chemically with another compound intentionally introduced in the vicinity of the toxin molecule holds the greatest promise for rapid and effective removal or inactivation of aflatoxin. Among such techniques are the use of chemosorbents and ammoniation. Other than the demonstrated reduction in bioavailability of aflatoxin as a result of hydrated sodium calcium aluminosilicate binding (Phillips *et al.*, 1988), ammoniation is the only chemical inactivation process that has been shown to destroy aflatoxin efficiently in cottonseed and cottonseed meal, peanuts and peanut meal and maize (Park *et al.*, 1988; Park & Price, 2001).

3.2.1 *Aflatoxin-binding agents*

Adsorption of aflatoxin using activated carbons, clays and aluminosilicates has been demonstrated in a number of studies. Bentonite clays (Masimango *et al.*, 1979) and activated charcoal (Decker, 1980), both used in oil purification, can adsorb up to 92% of aflatoxin present. A phyllosilicate clay currently used as an anti-caking agent has been shown to bind aflatoxin tightly and diminish markedly its uptake into the circulatory system, preventing aflatoxicoses and reducing levels of aflatoxin M_1 in milk (Phillips *et al.*, 1988). The specificity of the clays and their potential for binding nutrients in addition to aflatoxin remains a concern. Further research is required to determine whether these materials can be used in human foods.

3.2.2 *The ammoniation process*

The ammoniation process has been used to reduce aflatoxin levels in feed ration components in order to prevent the presence of aflatoxin in tissues and animal products such as milk. In the USA, the States of Arizona, California and Texas permit the ammoniation of cottonseed products. Texas has, in addition, approved the ammoniation procedure for aflatoxin-contaminated corn, but the treated corn may only be used in finishing beef cattle diets. Ammoniation is used in Brazil, France, Mexico, Senegal, Sudan and some states of the USA to lower aflatoxin contamination levels in animal feeds. The two procedures in widespread use are: (*a*) a high-pressure and high-temperature process (HP/HT) used at treatment plants, and (*b*) an atmospheric-pressure and ambient-temperature procedure (AP/AT) that can be used on the farm (Table 2). The HP/HT process

Table 2. Parameters and applications of ammonia procedures for aflatoxin decontamination

	Process	
	High pressure/high temperature	Atmospheric pressure/ ambient temperature
Ammonia level (%)	0.2–2	1–5
Pressure (psi)	35–50	Atmospheric
Temperature (°C)	80–120	Ambient
Duration	20–60 min	14–21 days
Moisture (%)	12–16	12–16
Commodities	Whole cottonseed, corn, cottonseed meal and peanuts	Whole cottonseed, corn
Application	Feed mill	Farm

Adapted from Park (1993b)
1 psi = 6.9 kPa

involves spraying the contaminated product with anhydrous ammonia (or introduction of ammonia gas) and water in a contained vessel. The treatment conditions, i.e. amount of ammonia (0.5–2%), moisture (12–16%), pressure (35–55 psi [240–380 kPa]), time (20–60 min) and temperature (80–120 °C) vary according to the initial levels of aflatoxin in the product (Park & Price, 2001).

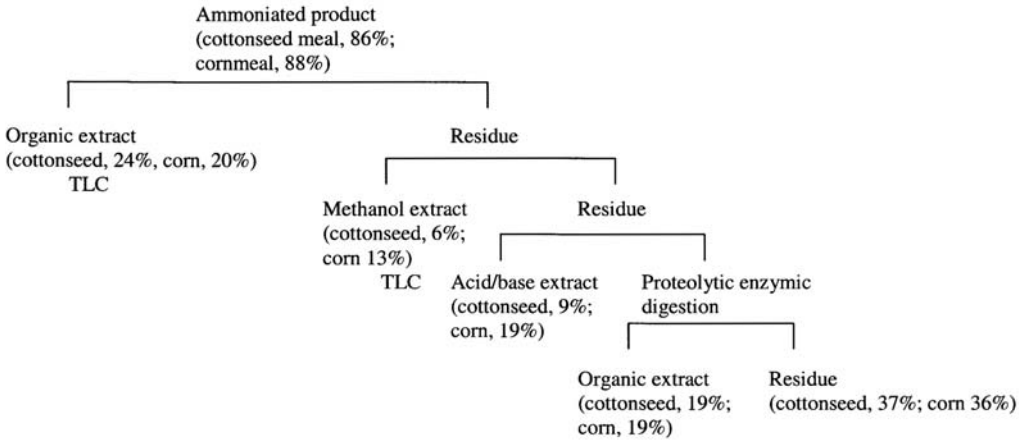
The AP/AT process also uses anhydrous ammonia and water, but the commodity is packed in a plastic silage-type bag or more recently simply covered with a tarpaulin and sealed. The sealed container is then held for 14–42 days, depending on the initial aflatoxin levels and the ambient temperature (25–40 °C); higher aflatoxin levels and a lower ambient temperature require a longer holding time. Similarly, the amount of ammonia (1–5%) can be varied according to the initial level of aflatoxin present and the moisture content of the material to be treated. Completion of treatment has to be predetermined in the HP/HT process, whereas with the AP/AT technique, the bag is probed and tested periodically until the results show that aflatoxin levels are equal to or below 20 ppb (Park & Price, 2001).

The addition of a formaldehyde anti-caking agent can improve the process (Prevot, 1986) and leads to no changes in milk production and composition (Calet, 1984). However, the addition of formaldehyde is not recommended in view of potential human exposure (see IARC, 1995).

(*a*) *Aflatoxin/ammonia chemistry*

Sequential fractionation of meals spiked with uniformly ^{14}C-labelled aflatoxin B_1 (Park *et al*., 1984) has allowed the partial isolation (Figure 2) and identification

Figure 2. Scheme for isolation and approximate concentrations of aflatoxin–ammonia reaction products in cottonseed and corn meals



From Park & Price (2001)
TLC, thin-layer chromatography

(Figure 3) of some of the decomposition products formed as a result of the aflatoxin–ammonia reaction. Hydrolytic scission of the aflatoxin lactone ring — the first step in the reaction — readily occurs under basic conditions, but is reversible when the ammoniation process is carried out under less drastic conditions. Under HP/HT and well controlled AP/AT conditions, the reaction proceeds to low-molecular-weight compounds, among them aflatoxin D_1 (molecular weight 286) and others of molecular weight 256 and 236 (Park *et al*., 1988).

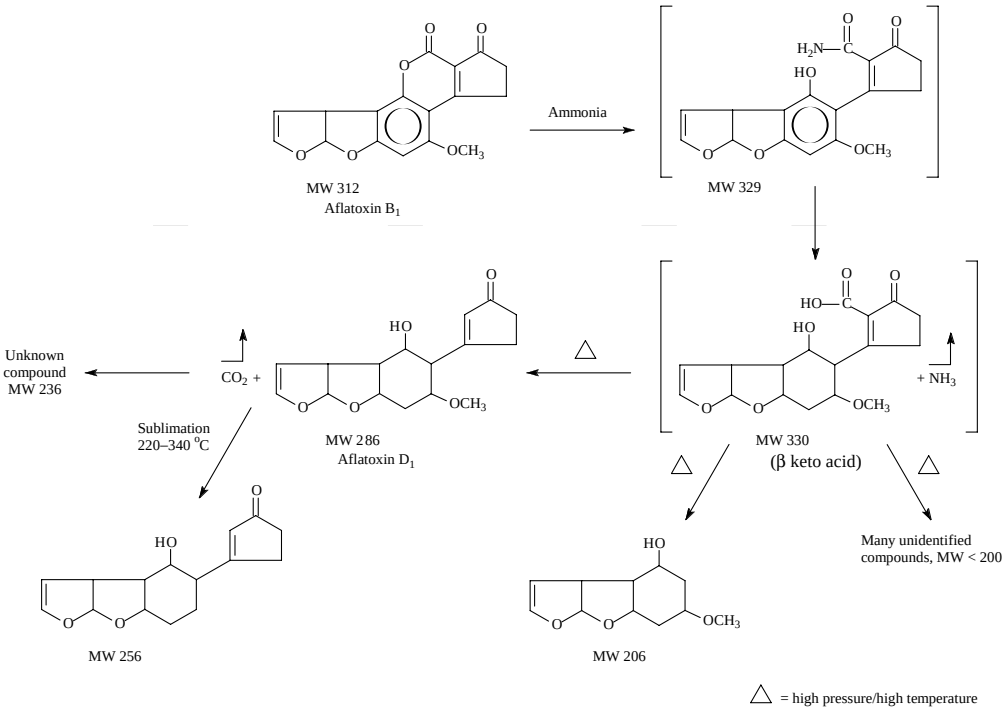
(*b*) *Efficacy of aflatoxin decontamination using ammonia*

Studies with peanuts, cottonseed and corn have demonstrated the effectiveness of the ammoniation process (Dollear *et al*., 1968; Brekke *et al*., 1977; Park *et al*., 1984; Martinez *et al*., 1994; Neal *et al*., 2001). Other studies are summarized in Table 3.

(*c*) *Safety of ammoniated commodities*

In vitro, sub-chronic and chronic studies have shown no mutagenic or tumorigenic lesions or toxic effects related to the HP/HT ammoniation procedure. Metabolism studies suggest poor absorption of decontamination reaction products compared with aflatoxin B_1. Livestock feeding studies with feedlot beef, dairy cattle, poultry, turkeys and ducklings have shown that the toxic effects observed following exposure to aflatoxin contamination are absent after ammoniation. These studies have been reviewed (Park *et al*., 1988; Park & Price, 2001).

Figure 3. Proposed formation of aflatoxin-related reaction products following exposure to ammonia



From Park *et al.* (1988)
Relative abundance and formation of reaction products are dependent on the conditions of ammoniation used.

Lactating mammals that ingest aflatoxin B_1 deposit the 4-hydroxylated metabolite, aflatoxin M_1, in their milk. Since infants and children have potentially greater vulnerability and sensitivity than adults, monitoring levels of aflatoxin M_1 in milk is important. Among human foods of animal origin, the rate of feed-to-tissue transfer of aflatoxin is highest for milk (Tables 4 and 5). Therefore, aflatoxins or their metabolites are not found in edible tissues except milk from animals fed aflatoxin-contaminated feed. The conversion rate of aflatoxin B_1 to aflatoxin M_1 in milk has been shown to vary between 1.1 and 14.7% for dairy rations containing between 20 and 800 ppb aflatoxin B_1 (Price *et al*., 1982; Fremy & Quillardet, 1985; Bailey *et al.*, 1994). It is higher than 1% when the amount of ingested aflatoxin B_1 is low. The primary purpose of the ammoniation procedure was to reduce aflatoxin M_1 residues in milk, and numerous studies have demonstrated its efficacy in eliminating aflatoxin residues in milk (Price *et al.*, 1982; Fremy *et al*., 1987; Bailey *et al.*, 1994).

Overall, decontamination reaction products in the feed matrix are usually derived from < 1% of the original aflatoxin content and large portions of these products are

Table 3. Studies on reduction of aflatoxin content by ammoniation of commodities

Commodity	Investigators	Initial AFB_1 content (ppb)	Process	Process parameters	Final AFB_1 content (ppb)	Reduction (%)
Corn	Hughes *et al*. (1979)	754 total [603 AFB_1]	AP/AT	1% NH_3, 18% m, 12–13 days	3.5	> 99
	Weng *et al*. (1994)	7500	HP/HT	2% NH_3, 16% m, 55 psi, 40–45 °C, 60 min	517	93
	Weng *et al*. (1994)	7500	HP/HT	2% NH_3, 16% m, 17 psi, 121°C, 60 min	31	> 99
Cottonseed	Jorgensen & Price (1981)	800 total	AP/AT	2% NH_3, 7.5% m, 21 °C, 15 days	< 20	99
	Bailey *et al*. (1994)	5200	AP/AT	1.5 % NH_3 17% m, 42 days	< 10	> 99
	Bailey *et al*. (1994)	1200	HP/HT	4% NH_3, 14% m, 40 psi, 100 °C, 30 min	ND (≤ 0.1)	~> 99
Peanut meal	Gardner *et al*. (1971)	121 total	HP/HT	NH_3 concentration not specified, 15% m, 30 psig, 93 °C, 15 min	ND	~> 99

AFB_1, aflatoxin B_1; m, moisture; AP/AT, atmospheric pressure/ambient temperature; HP/HT, high pressure/high temperature; ND, not detected; psig, pound per $inch^2$ gauge (indicating the pressure above atmospheric pressure); 1 psi = 6.9 kPa

Table 4. Relation of aflatoxin levels in feed to aflatoxin residue levels in edible tissues

Animal	Tissue	Aflatoxin	Feed:tissue ratio[a]
Beef cattle	Liver	B_1	14 000
Dairy cattle	Milk	M_1	75
		Aflatoxicol[b]	195 000
Swine	Liver	B_1	800
Layers	Eggs	B_1	2200
Broilers	Liver	B_1	1200

From Park & Pohland (1986); Park & Stoloff (1989)
[a] Level of aflatoxin B_1 in the feed divided by the level of the specified aflatoxin in the specified tissue
[b] A metabolite of aflatoxin B_1

Table 5. Aflatoxin B_1 levels in feed components required to yield 0.1 ng/g residue levels of aflatoxins in edible tissues

Species	Contamination level of aflatoxin B_1 (ng/g) in rations			
	Corn	Peanut meal	Cottonseed meal	Cottonseed
Beef cattle	1800	1400	12 725	14 000
Dairy cattle	14	54	54	38
Swine	105	730	1600	–
Layer	325	1835	2445	–
Broiler	180	925	1200	–

From Park & Pohland (1986)

strongly bound to feed components so that they are biologically unavailable to the animals (Park *et al*., 1984) or are eliminated by excretion (Hoogenboom *et al.*, 2001).

4. Outstanding Health Questions in Aflatoxin Management

Increased liver cancer incidence associated with aflatoxins occurs in areas of the world with chronic high levels of toxins, frequently exceeding the regulatory levels under consideration by the Codex Alimentarius Commission (2001) by large amounts, sometimes factors of 10 or 100, and endemic infection with hepatitis B or C viruses (HBV or HCV). Some basis exists for quantifying the effects of aflatoxin exposure on liver cancer risk, and for the greater impact of aflatoxins in areas of high HBV or HCV

incidence (JECFA, 2001; see Section 5). However, uncertainty remains regarding (1) the health effects of occasional high exposures occurring due to unusual weather patterns, in comparison with those due to normal chronic exposure; (2) the effect of combinations of mycotoxins, e.g. aflatoxins and fumonisins, on cancer risk; and (3) the broader health effects of aflatoxin exposure. Under the latter consideration, the most important aspects are (1) the greater sensitivity of children to acute toxicity of aflatoxins as compared with adults; (2) the in-utero effects of aflatoxins, known to cross the placenta; (3) the immunosuppressive effects of aflatoxins, which may influence susceptibility to infectious disease; (4) the suppressive effects of aflatoxins on growth; and (5) the interactions between HCV, HBV and aflatoxins in liver cancer development in various populations in the world.

The areas mentioned are important ones for future research, to provide information of direct relevance to public health decisions regarding aflatoxin exposure.

5. Previous Recommendations

Although some countries with fully developed market economies have enforced regulations on aflatoxins for some 30 years, it is only recently that a broader consensus has been developed on the impact of aflatoxins and other agriculturally important toxins on human populations. The health effects include those resulting from exposure in foods, from reductions in the quality of the crops affected and from reduced animal production. The health consequences of lowered income are also pertinent in some communities. This consensus was reached when the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (JECFA) established an hypothetical standard for aflatoxins (JECFA, 1998), provisional maximum tolerable daily intakes (PMTDIs) for deoxynivalenol and fumonisins and a provisional tolerable weekly intake (PTWI) for ochratoxin A (JECFA, 2001). For aflatoxin and fumonisin, exposure in excess of the PMTDI occurs in much of Africa as well as parts of Asia and South America.

JECFA attempted to separate the portion of risk of liver cancer attributable to aflatoxin from the risk attributable to other factors (JECFA, 1998, 2001). JECFA modelled dose–response curves from laboratory animal studies as well as human epidemiological studies of aflatoxin carcinogenicity. The risk of liver cancer from aflatoxin consumption was significantly higher (perhaps 30-fold) in individuals who were chronically infected with hepatitis B virus (hepatitis B surface antigen (HBsAg)-positive) than in those who were not (HBsAg-negative).

It was concluded that populations such as those in western Europe and the USA with a low prevalence of HBsAg-positive individuals or populations with a low mean intake of aflatoxins are unlikely to achieve a decrease in liver cancer cases from more stringent

aflatoxin standards (such as lowering the regulatory hypothetical standard for aflatoxin B_1 in peanuts from 20 μg/kg to 10 μg/kg) (JECFA, 2001).

Populations with a high prevalence of HBsAg-positive persons and high aflatoxin intake would benefit from reductions of aflatoxin intake, and such a reduction in aflatoxin intake could be achieved without loss of valuable food sources and risk of malnutrition or starvation (JECFA, 1998).

The FAO/WHO/UNEP (United Nations Environment Programme) (1999) conference, representing 38 countries and 10 international organizations, made a series of recommendations reflecting a new perspective on the health and economic impact of mycotoxins in staple foods. It was recommended that:

- Surveillance should be targeted to staple foods.
- HACCP (hazard analysis: critical control point) principles can be used to highlight the roles that fungal ecology, crop physiology and agronomic practices play in mycotoxin contamination prevention and control.
- The stakeholders in the production chain, particularly farmers, should be made aware of the importance of measures to reduce mycotoxin contamination.
- Before recommending the introduction of crops or new genotypes into new environments, consideration should be given to the potential for increased [toxigenic] fungal infections.
- Training programmes for the development of practical control and management strategies should be [developed and] conducted in developing countries in order to set up strong mycotoxin management programmes.

6. Trade in Crops

Mycotoxin contamination in crops is a major determinant of trade for all economies. Typically, the higher-quality grain or nuts are traded and the poorer-quality product remains with the farmer for consumption at the village level or for use in animal production. Major risks exist in this situation, especially where unseasonable rains or other climate variations result in excessive mycotoxin contamination of crops, where consumer standards for urban customers increase, or where commodities intended for international trade are upgraded by sampling or sorting.

Where industrialized domestic animal production takes place, there are a number of strategies to make use of crops with undesirable mycotoxin concentrations. These include dilution with other feed ingredients, addition of absorbents for aflatoxins, additions of sugar and, where permitted, ammoniation. In developing countries, however, diverting grains contaminated with aflatoxins into rural animal protein production may result in a substantial reduction in feed conversion as well as increased herd or flock mortality and morbidity. The economic impact of such reduced feed conversion can eliminate the expected increased return on investments in animal protein production.

Furthermore, the reduced animal production increases protein energy malnourishment in vulnerable groups, e.g. children and pregnant women.

7. Conclusions

This Annex has dealt in general terms with a number of very complex issues. Many areas have been highlighted which are in need of much future research to provide information that is directly relevant not only to public health, but also to availability of a wholesome food and feed supply worldwide. The following general conclusions and recommendations emerged from the deliberations of the Working Group.

1. Limiting aflatoxin occurrence in crops before harvest can be achieved by limiting drought and high temperature stress, controlling weeds, reducing insect damage, using effective harvesting techniques and reducing *Aspergillus* spore numbers in soil by crop rotation.
2. Genetic engineering may offer new ways of limiting the pre-harvest contamination of some crops by aflatoxins.
3. Aflatoxins can be controlled in susceptible crops after harvest by controlling factors which affect fungal growth, e.g. water activity, temperature, gas atmospheres, and the use of insecticides or preservatives. The prime consideration for storage of grains and nuts is to maintain the water activity (by control of moisture content) below the limit for fungal growth.
4. Aflatoxin levels can be reduced in stored commodities by physical means, such as colour sorting, density flotation, blanching and roasting.
5. Where approved, aflatoxin levels in commodities destined for animal feeds can be reduced by chemical processes. Such processes include agents which bind aflatoxins, such as adsorbent clays, and the ammoniation process. The main use for ammoniation is in elimination of aflatoxin from feed for dairy cows.
6. Increased liver cancer incidence associated with aflatoxin exposure occurs in areas of the world where chronic high levels of aflatoxins (often many times higher than regulatory limits) and endemic infection with HBV or HCV occur together. Populations with a low prevalence of HBV chronic carriers and a low mean aflatoxin intake are unlikely to achieve a decrease in liver cancer cases by introducing lower aflatoxin limits. In contrast, in populations with a high prevalence of HBV chronic carriers and high exposure to aflatoxin, measures to reduce aflatoxin exposure would be desirable and beneficial.

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