PLACENTAL LOCALISATION OF *ASPERGILLUS FUMIGATUS* IN BOVINE MYCOTIC ABORTION: ENHANCEMENT OF SPORE GERMINATION *IN VITRO* BY FOETAL TISSUE EXTRACTS

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ABORTION associated with mycotic placentitis is well known in cattle and causes considerable economic loss in Britain (Austwick and Venn, 1961; Hugh-Jones and Austwick, 1967). Occasionally foetal infection occurs but the dam usually shows no ill effects, spontaneously recovers after aborting, and may subsequently conceive normally (Ainsworth and Austwick, 1959). Although numerous fungi have been implicated in the disease (Ainsworth and Austwick, 1959; Campbell, 1969) *Aspergillus fumigatus* is the major causal organism, accounting for over 60% of cases in Britain and the USA (Austwick and Venn, 1961; Hillman, 1969).

Intravenous inoculation of the conidia of *A. fumigatus* into sheep and cattle led to abortion with striking localisation of hyphal growth in the placenta (Cysewski and Pier, 1968; Hillman and McEntee, 1969). Extra-placental lesions may develop after intravenous inoculation but these either remain small or rapidly disappear whereas infection persists and spreads throughout the placenta of both the cow (Hill *et al.*, 1971) and sheep (Pier, Cysewski and Richard, 1972).

Tissue specificity of animal infections may be governed by nutritional factors or local host defences or both (Pearce and Lowrie, 1972). Erythritol enhances the growth of *Brucella abortus* and is concentrated in the placenta of cattle and other animals susceptible to contagious abortion. Its presence in susceptible tissues appears to determine the localisation of the pathogen (Keppie, 1964; Smith, 1968). Other bacterial pathogens cause localised infections in the ungulate placenta yet are not affected by erythritol; other nutrients or host defences may be involved in their placental localisation (Pearce and Lowrie, 1972). In plants host and tissue specificities of fungal infections have been explained by the natural distribution of appropriate nutrients, for example, *myo*-inositol in plum trees (Lukezic and Devay, 1964) and choline and betaine in anthers of wheat (Strange and Smith, 1971a; Strange, Majer and Smith, 1972; Strange, Smith and Majer, 1972). Other examples have been explained by differences in host defences (Kuć, 1972).

This paper reports a survey of extracts of bovine tissues for their ability to enhance hyphal extension of *A. fumigatus in vitro*. The reasons for the

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observed effect have been investigated and the possible role of such enhancement in the localisation of infection *in vivo* is discussed. A preliminary report has already appeared (White and Smith, 1972).

MATERIALS AND METHODS

A. fumigatus. A subculture of the strain of Cysewski and Pier, isolated from an aborted bovine foetus and pathogenic for sheep, was supplied by Dr C. K. Campbell.

Spore suspension. Conidia were washed from 3-4 day cultures on malt-agar slopes with sterile aqueous Tween 80 (0.05% v/v), washed in two changes of sterile distilled water, sedimented by centrifugation (1000g) and re-suspended in sterile distilled water to a final concentration of $6-7 \times 10^6$ conidia per ml. Suspensions were stored at 4°C and when required the spores were re-suspended and the concentration adjusted to 10^6 conidia per ml.

Tissue extraction. Tissues from eight pregnant cows were removed and stored at -20°C until required. Chopped tissues, mixed with an equal weight of distilled water, were homogenised at 4°C in a Sorvall Omnimixer for $3 \times \frac{1}{2}$ min. with 2-min. intervals. After centrifugation at 30,000g for 120 min. the supernatant from each homogenate was adjusted to pH 7.5, distributed in 2-ml aliquots and stored at -20°C.

Phosphate-buffered saline (pH 7.3). This was Dulbecco A Buffer (Oxoid).

Hyphal-extension assay. This assay detects both effects on spore germination and effects on hyphal growth. The method was that of Strange and Smith (1971b) with modifications of agar medium, time and temperature of incubation, and number of spore-containing wells. Czapek-Dox Agar (Oxoid) was dispensed in 5-ml volumes into glass petri dishes of 5.1-cm diameter. A pattern of six wells, each 0.5 cm in diameter and surrounding a central well, was cut into each plate. All wells were 1.0 cm apart (centre to centre). Extract (20 µl) was placed in the central well and spore suspension (10 µl) was placed in each outer well. After incubation at 37°C for 17 hours, each plate was examined microscopically and the maximum distance that the hyphae from each outer well had grown towards the central well was measured. The average percentage increase of hyphal extension over control plates with no extract was calculated. An extract of foetal cotyledon from cow no. 1 was taken as the standard, and the relative activities of the other extracts were expressed as the ratio of the dilution of standard producing 35% enhancement of hyphal extension over the control to the dilution of test extract producing the same degree of enhancement.

Influence of extracts on mycelium dry weight in shake culture. Liquid Czapek-Dox Medium (Oxoid) was dispensed in 9-ml volumes into 100-ml conical flasks and supplemented with either distilled water or tissue extract (1.0 ml) previously sterilised by membrane filtration (0.22 µm pore size; Millipore). Flasks were seeded with 1.0-ml spore suspension and incubated in a Gallenkamp orbital incubator at 37°C and 150 r.p.m. After fixing with formalin (2% v/v final concentration) the mycelium was collected on tared glass-fibre filters, washed with distilled water, and dried to constant weight at 105°C.

Influence of extracts on spore germination. Spores were suspended, in liquid Czapek-Dox Medium plus water or extract, to a final concentration of 10^6 per ml. Drops were placed on clean circular coverslips and incubated at 37°C in a humid plastic container. At intervals of 5 and 6.25 hours, coverslips were removed and the spores stained by adding a drop of trypan blue. Germination was scored by microscopic examination, a spore being considered to have germinated when it bore a germ tube at least as long as it was broad (Manners, 1966). Three coverslips were examined at each time; 200 spores were counted on each and the mean percentage germination was calculated.

Gel filtration. Extracts were separated into "high molecular weight" (HMW) and "low molecular weight" (LMW) fractions by filtration through G-25 Sephadex (coarse grade). One millilitre of extract was run into gel prepared in phosphate-buffered saline in a column 25 cm long $\times$ 1.5 cm diameter (25-ml bed volume). The first 2.5 ml of eluant was discarded, the next 11 ml was the HMW fraction and the next 20 ml the LMW fraction.

RESULTS

Hyphal-extension assay

Extracts of most foetal and maternal tissues examined from eight cows produced some enhancement of hyphal extension (table I) and the degree of enhancement was linearly related to log dilution (fig. 1). However, extracts of some foetal livers and the allantoic and amniotic fluids showed inhibition of the

TABLE I

Hyphal-extension assay: relative activities of aqueous extracts of bovine tissues

Tissues	Relative activity* of tissue extract of cow number							
	1	2	3	4	5	6	7	8
Foetal								
cotyledon	1.0 (1.8)	0.5	0.7	1.0	0.7	0.7	0.6	1.5
chorion	0.6 (0.7)	0.3	0.5	0.1	0.3	0.4	...	...
lung	0.5 (0.2)	0.3	0.2	0.2	0.3	0.5	...	...
heart	0.4 (0.3)	0.3	0.4	0.2	0.3	0.4	...	...
spleen	...	0.4	0.2	0.4	0.3	0.5	...	...
liver	0.1 (0.7)	0.4	0.1	...	0.3	0.1	...	...
kidney	0.6 (0.8)	0.4	0.2	...	...	...	...	...
muscle	0.4 (0.1)	0.1	0.1	0.4	0.1	0.3	...	...
allantoic fluid	...	0.4	0.1	0.7	0.8	...	...	...
amniotic fluid	1.6 (1.6)	0.1	0.1	0.2	1.3	...	...	...
Maternal								
caruncle	0.3 (0.3)	0.3	0.3	0.4	...	...	0.2	0.4
uterus	0.2 (0.1)	0.5	0.3	0.6	0.4	0.6	0.1	0.8
lung	...	<0.1	<0.1	...	...	...	<0.1	<0.1
heart	...	0.1	0.1	...	...	...	...	...
spleen	0.2 (0.3)	0.7	0.4	...	...	...	0.8	0.5
liver	0.01 (0.01)	0.01	0.01	...	...	...	...	...
kidney	0.1 (0.2)	0.2	0.1	...	...	...	...	...
muscle	0.2 (0.1)	0.3	0.01	...	...	...	...	...

* Ratio of dilution of cotyledon extract of cow no. 1 that gave a 35% enhancement. Activities of LMW fractions are given in parentheses. Pooled calf serum was inactive and pooled foetal calf serum had a relative activity of 0.1.

... = Tissues not obtained.

enhancement at low dilution and the dose-response curves of the less active extracts tended to have shallower slopes (fig. 1). Extracts of foetal tissues showed high activity, and in six of the eight cows foetal cotyledon extracts were the most active. Foetal fluids varied considerably in their activity. Maternal-tissue extracts had, in general, low activity except for the caruncles, uterus and spleen in some animals. The active material was found in the LMW fractions but erythritol, as well as inositol, choline, betaine, fructose, and urea, were inactive when tested at 0.5–1000 μg per ml.

Shake culture

Similar growth rates were observed in both unsupplemented medium and in medium plus cotyledon extract (fig. 2). This prompted an investigation of

FIG. 1.—Enhancement of hyphal extension of *Aspergillus fumigatus* by extracts of foetal cotyledon (A), maternal muscle (B) and maternal liver (C) from cow no. 1.

FIG. 2.—Growth of *Aspergillus fumigatus* in liquid Czapek-Dox medium supplemented with 0.05% extract of cotyledon from cow no. 1 (A), and unsupplemented (B).

FIG. 3.—Hyphal-extension activity of extract of cotyledon from cow no. 1 on normal spores (A) and pre-germinated spores (B).

TABLE II

Germination of Aspergillus fumigatus spores in liquid Czapek-Dox Medium supplemented with 0.2% (v/v) of tissue extracts of cow no. 1

Tissue-extract supplement	Mean percentage germination at	
	5 hours	6.25 hours
Foetal cotyledon	6.7 (2.3)*	45.1 (3.2)
chorion	4.3 (2.0)	41.3 (5.1)
lung	0.5 (0.2)	35.2 (5.7)
heart	1.3 (0.3)	17.7 (1.1)
liver	1.5 (0.6)	31.8 (3.0)
kidney	1.3 (0.6)	30.5 (4.4)
allantoic fluid	5.6 (0.8)	53.6 (3.4)
Maternal caruncle	1.2 (0.7)	22.8 (2.5)
uterus	<0.5	4.5 (0.3)
spleen	<0.5	12.4 (3.8)
liver	<0.5	<0.5
kidney	<0.5	6.4 (1.0)
muscle	<0.5	9.8 (1.6)
Control	<0.5	3.6 (0.8)

* Standard deviation of mean.

the possibility that extracts were enhancing spore germination rather than hyphal growth.

Effect on germination

Spores were applied to plates as in the normal hyphal-extension assay, but no tissue extract was placed initially in the central well. The plates were incubated at 37°C and examined microscopically at intervals. When germ tubes were observed on the spores (c. 8 hours) cotyledon extract (cow no. 1) was added and the incubation continued for a further 9 hours. Under these conditions, cotyledon extract was no longer active in enhancing hyphal extension (fig. 3). When similarly tested, none of the tissue extracts of cow no. 1 enhanced hyphal growth from pre-germinated spores.

A direct effect on germination was then examined in supplemented Czapek-Dox medium (table II). Foetal-tissue extracts, notably cotyledon extract, had the greatest enhancing effect on the rate of germination.

DISCUSSION

The natural portal of entry of fungal elements in bovine mycotic abortion is unknown. However, spores probably reach the placenta via the maternal blood since intravenous inoculation is the only successful method of experimental infection (Gilman and Birch, 1925; Bendixen and Plum, 1929; Hillman and McEntee, 1969; Hill *et al.*, 1971). The placenta seems to be the only organ which provides a suitable environment for rapid vegetative growth of *A. fumigatus* (Hill *et al.*, 1971) and we have demonstrated that placental extracts stimulate germination of *A. fumigatus* spores *in vitro*. However, this activity was not confined to extracts of the foetal placenta. Extracts of other foetal tissues and extracts of some caruncles and uteri showed activity, though to a lesser extent. The active material may have originated in the foetal placenta and passed to these tissues *in vivo*. Other maternal tissues produced extracts of low activity, with the exception of the spleen. The significance of the high splenic activity is unknown, and only when the placental material is identified can we know whether it is also present in this tissue.

The germination-stimulating material in the placenta may be important in determining the latter's special susceptibility to fungal infection. Germinated spores of *A. flavus* seemed more able than dormant spores to overcome the defence mechanisms of the mouse when administered intraperitoneally (Sidersky *et al.*, 1972). Rapid germination and growth in the bovine placenta might thus assist in overcoming local host defences. This might lead to the production of emboli, similar to those produced in the ovine placenta by latex spheres (Cysewski and Pier, 1968), and hence to a progressive placental infection. The reason why excessive fungal growth does not occur in other tissues that contain germination-stimulating material (notably the maternal spleen) may be that it is prevented by host defence-mechanisms which are possibly lacking in the placenta (Pearce and Lowrie, 1972). In this context it is of interest that reducing the defence mechanisms with cortisone rendered mice more

susceptible to infection with aspergillus spores (Bhatia and Mohapatra, 1969, 1970; Sidransky *et al.*, 1972). Alternatively, active material may not be available to spores in the insusceptible tissues *in vivo*.

This work has given an indication that, by stimulating germination, nutritional factors may play a role in the susceptibility of the placenta to *A. fumigatus* infection. However, the nature and distribution of the active material, which appears to be of low molecular weight, must be elucidated to support this suggestion.

SUMMARY

Aspergillus fumigatus shows a marked affinity for the bovine placenta in mycotic abortion. Aqueous extracts of foetal tissues and maternal uterus and caruncles from eight pregnant cows enhanced hyphal extension of *A. fumigatus in vitro*, and the foetal cotyledon extracts were the most active. Extracts of other maternal tissues had low activity with the exception of the spleen. The enhancement appeared to be due to accelerated germination, and the active material was of low molecular weight. It may play a part in the placental localisation *in vivo*.

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