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Annual Ryegrass Toxicity

Aetiology, Pathology and Related Diseases

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Contents

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1. Introduction

Several livestock poisoning outbreaks in Australia, involving neurological signs and characterised by high mortalities, are now known to be, or are suspected of being, caused by naturally occurring mixtures of toxic tunicaminyluracil antibiotics. These potent inhibitors of protein glycosylation are produced by certain *Streptomyces* and by *Clavibacter toxicus* and possibly other bacteria.

Each species of bacterium appears to synthesise a characteristic mixture of highly toxic antibiotics (Cockrum and Edgar, 1983; Eckardt, 1983). The different toxin mixtures have been given different trivial names which reflect their microbial source and composition. Tunicaminyluracil toxin complexes which have been characterised include the corynetoxins, produced by *Clavibacter toxicus* (previously *Corynebacterium* sp.), the tunicamycins produced by *Streptomyces lysosuperificus*, antibiotic 19290 from an unnamed *Streptomyces* sp. and the streptovirudins which are products of *Streptomyces griseoflavus* (Cockrum and Edgar, 1983). Other tunicaminyluracil antibiotic mixtures are known, e.g. mycospocidin and antibiotic 24010, but these have only been partially characterised (Eckardt, 1983).

Not many of the tunicaminyluracil antibiotic mixtures/complexes recorded in the literature are known to cause poisoning of livestock in the field. Most episodes of poisoning, where the nature of the toxins have been determined, have been caused by the corynetoxins produced by *C. toxicus*. Thus the corynetoxins are responsible for annual ryegrass toxicity (ARGT), floodplain staggers and Stewart's Range syndrome in Australia.

2. Background

Nematodes of the genus *Anguina* can carry plant-pathogenic *Clavibacter* sp. bacteria into the developing seedheads of several grass species where the bacteria establish colonies in galls induced by the nematodes (Thorne, 1961). Between 1945 and 1961 a neurological disease was described in livestock fed the screenings of infected fescue grass (*Festuca nigrescens*) in Oregon, USA (Haag, 1945; Shaw and Muth, 1949; Galloway, 1961). Sheep and cattle died after showing tremor, ataxia and convulsions. No significant pathological findings were described in affected livestock but ingestion of the toxic grass produced haemorrhages, congestion and oedema of extremities, and death in laboratory rats. The primary source of the toxicity and the nature of the toxins were not identified and remain unknown.

Outbreaks of an apparently similar neurological disease in sheep and cattle grazing *Anguina* sp. – *Clavibacter* (*Corynebacterium*) sp. infected annual ryegrass were subsequently reported from Australia. In 1967 annual or Wimmera ryegrass (*Lolium rigidum*) toxicity was first described in South Australia (McIntosh *et al.*, 1967) and in 1971 the disease was also recorded in Western Australia (Gwynn and Hadlow, 1971). The toxicity was shown to be associated with the bacterium (Lanigan *et al.*, 1976; Payne *et al.*, 1983) which was initially identified as a *Corynebacterium* sp. and later named *Clavibacter toxicus* (Riley and Ophel, 1992). The toxins (corynetoxins) were identified as a novel mixture of tunicaminyluracil antibiotics (Edgar *et al.*, 1982; Frahn *et al.*, 1984). Initially the disease was confined to a few properties, but in subsequent years it has spread over extensive areas of both States (Australian Bureau of Statistics, 1989). Evidence suggests spread of the causal bacterium is likely to continue. The nematode vector, *A. agrostis* (*funestra*), has been found over a large area in Victoria but as yet ARGT has not occurred there.

A disease of long-standing in the southeast of South Australia, Stewart's Range syndrome, has recently been shown to be caused by bacterially infected annual beard grass (Finnie, 1991) and a major outbreak of an apparently new poisoning disease (floodplain staggers) in north-western New South Wales in the summer of 1990 in which more than 1500 cattle, 2000 sheep and a number of horses died, has recently been shown to be caused by ingestion of infected blowaway grass (*Agrostis avenacea*) (E.O. Davis, pers. comm. 1991). In both instances the toxins (J.A. Edgar, unpublished data 1991) and bacterium (A.C. MacKay, pers. comm. 1991) involved have been shown to be the same as those causing ARGT; however, the nematode vector differs from that associated with ARGT (A.C. MacKay, pers. comm. 1991).

An outbreak of poisoning of pigs being fed microbially contaminated, water-damaged wheat (Bourke, 1987) has also been shown to be caused by toxic tunicaminyluracil antibiotics (Cockrum *et al.*, 1989). A nematode appears not to have been involved in this case and the microorganism producing the toxins was not identified. The unique mixture of toxins found, however, suggests that a novel microbial source was involved (Cockrum *et al.*, 1989).

ARGT, floodplain staggers and Stewart's Range syndrome are examples of corynetoxin poisoning of which ARGT is, at present, the most widely occurring and the most studied.

3. Etiology of Annual Ryegrass Toxicity

3.1. Role of the Casual Organisms

Annual ryegrass which becomes toxic is colonised during tillering by infective larvae of the nematode *Anguina agrostis* (Price, 1973; Stynes and Bird, 1980). As the grass matures, floret

primordia are modified by the nematodes which feed on the plant and hollow, flask-shaped, galls are produced (Price, 1973; Price *et al.*, 1979; Stynes *et al.*, 1979). In some galls the life cycle of the nematode is completed and the flask-shaped structures become filled with the next generation of infective larvae.

Other galls, initially induced by the nematode, become filled with yellow masses of a proliferating bacterium, *Clavibacter toxicus* (Price, 1973; Bird and Stynes, 1977; Riley and Ophel, 1992), carried into the plant firmly attached to the cuticle of the nematode (Price, 1973; Bird and Stynes, 1977). As the bacterium proliferates, yellow slime may ooze from the galls and become visible on external parts of the plant in early spring (Price, 1973; Stynes and Wise, 1980).

3.2. The Toxic Principle

Separated fractions of parasitised annual ryegrass plants have been fed to laboratory animals to determine the toxic component (McIntosh *et al.*, 1967; Lanigin *et al.*, 1976; Berry *et al.*, 1976; Stynes *et al.*, 1979). The toxicity was found to be concentrated in the wall of *C. toxicus*-infected galls whereas the nematode galls and other parts of the plant were non-toxic. Production of the corynetoxins *in vitro* by *C. toxicus* in the absence of nematode and plant material (Payne *et al.*, 1983; Payne and Cockrum, 1988) unequivocally confirmed the bacterial origin of the toxins.

A family of highly toxic glycolipids, called corynetoxins, was isolated from *C. toxicus*-infected galls and shown to be the etiological agent of ARGT (Vogel *et al.*, 1981; Edgar *et al.*, 1982; Frahn *et al.*, 1984). Corynetoxins belong to the tunicaminyluracil group of antibiotics (Edgar *et al.*, 1982; Frahn *et al.*, 1984). They inhibit N-glycosylation of proteins (Jago *et al.*, 1983) and this mode of action is likely to be, either directly or indirectly, a major factor in the biochemical pathogenesis of ARGT (Culvenor and Jago, 1985).

The antibiotic properties of corynetoxins provided the basis for a useful bacterial inhibition assay indicative of corynetoxins in pasture samples (Stynes and Vogel, 1983). The presence of at least 125 ng corynetoxins/mL extract inhibits the growth of the test organism, *Clavibacter tritici.*

High performance liquid chromatography methods have been developed for quantitation and confirmatory identification of the toxins (Cockrum and Edgar, 1985) and fast atom bombardment mass spectrometry, in association with catalytic hydrogenation to generate fully saturated derivatives, provides an unequivocal test of identity (e.g. Cockrum *et al.*, 1989).

Infected annual ryegrass remains toxic when stored at room temperature for several years and the toxicity is not reduced when dried at 100°C for three hours (Cockrum and Edgar, 1985; Culvenor *et al.*, 1978). Also, corynetoxins are not detoxicated by *in vitro* incubation with rumen fluid for up to six days (Vogel and McGrath, 1986).

4. Occurrence and Distribution

4.1. Occurrence

ARGT typically occurs in animals grazing infected annual ryegrass in pasture or cereal stubble between late spring and the end of summer. The syndrome has also been observed in livestock fed hay or cereal screenings which contained infected ryegrass (Berry and Wise, 1975). In Western Australia, outbreaks most frequently occur in pasture paddocks the year after they have been cropped.

Occasionally ARGT occurs in early winter when green feed is short and heavy stocking rates force animals to graze dry pasture residues.

4.2. Distribution in South Australia

ARGT and *C. toxicus* were first discovered in South Australia in 1956. In 1967 toxicity was reported to have occurred on nine farms in the area bounded by Minoora, Black Springs and Waterloo in the mid north of the State (McIntosh *et al.*, 1967). The disease has now spread to all the main cropping areas; to Eyre Peninsular, Kangaroo Island and up to the border with Victoria.

4.3. Distribution in Western Australia

ARGT was first recognised in Western Australia on a property near Gnowangerup in 1968. Since then the causal organisms have been identified on 934 farms where livestock losses have occurred (Australian Bureau of Statistics, 1989). Affected farms are located in a grazing and cereal growing region where cropping practices and an average annual rainfall of 300–500 mm have favoured establishment of dominant stands of annual ryegrass in the year after cropping. Significant factors, which in the past enhanced the proliferation of annual ryegrass and the associated parasites, are the increased used of fertilisers, shorter rotations, discontinued use of fallowing and a reduction in the burning of cereal stubbles (Stynes and Wise, 1980).

4.4. Distribution Elsewhere

Anguina agrostis has been found over a large part of western Victoria but *C. toxicus* and ARGT have not yet been recorded in Victoria. Two other forms of corynetoxin poisoning which have occurred in Australia , floodplain staggers and Stewart's Range syndrome, are caused by *C. toxicus*-infected blowaway grass (*A. avenacea*) and annual beardgrass (*Polypogon monspeliensis*) respectively (J.A. Edgar, unpublished data 1991). Stewart's Range syndrome has been seen in the southeast of South Australia regularly for more than twenty years (Finnie, 1991) while floodplain staggers was first recognised in the summer of 1990–91 (E.O. Davis, pers. comm. 1991).

A disease, clinically indistiguishable from ARGT, was reported from South Africa in 1980 (Schneider, 1981) and the toxins involved have been shown to be identical to the corynetoxins causing ARGT in Australia (Cockrum and Edgar, 1985).

An apparently related poisoning disease of livestock was seen in Oregon, USA, from 1945 to 1961. No recent outbreaks have been recorded. The bacterium involved and the toxins responsible remain unidentified (Galloway, 1961).

A poisoning of pigs, involving clinical signs indistiguishable from those seen in ARGT (Bourke, 1987), has also been shown to be caused by a novel mixture of tunicaminyluracil antibiotics of unknown microbial origin (Cockrum *et al.*, 1989).

4.5. Possible Means of Spread of Causal Organisms

In the case of ARGT, it may be assumed that factors which favour the spread of ryegrass seed would allow the dispersal of infected galls. Similar factors will be involved in the spread of other forms of tunicaminyluracil antibiotic toxicity. Probable means are the movement of farm machinery, hay and grass seed; as well as by wind and along water courses (Price, 1973; Berry and Wise, 1975). The possibility of galls adhering to fleeces, hides and hooves of animals should also be considered. As the available evidence suggests that few *Anguina* larvae survive passage through the alimentary tract of sheep (Price, 1973), it is unlikely that movement of sheep which have recently ingested infected ryegrass would be a significant mode of dispersal.

5. Clinical Signs

5.1. Livestock

Signs of ARGT and related diseases in sheep and cattle are similar, being characterised by intermittent bouts of neurological disturbance such as ataxia, collapse, tremor and convulsions often followed by death (McIntosh *et al.*, 1967; Berry and Wise, 1975).

At first, signs may only be observed if affected animals are stimulated. When an affected flock or herd is moved, a proportion of animals may stagger, tremble or collapse into ventral or lateral recumbency. Recumbent animals may remain down for 5–10 s and show no further signs but others may exhibit neck ventroflexion, opisthotonus, head nodding or swaying, and tetanic or clonic convulsions.

Affected animals which regain their feet may stagger away with a stiff-legged, jumping or swaying gait and appear normal. As the disease progresses neurological signs become more frequent with depression and ataxia giving way to almost continuous tremors and convulsions until death. Mortality rates vary up to 100%.

Abortions have been observed in pregnant ewes grazing toxic annual ryegrass. In plasma samples from affected sheep there are increased levels of liver specific enzymes such as ornithine carbamyl transferase and sorbitol dehydrogenase (Berry *et al.*, 1982). Levels of uridine diphospho-N-acetylglucosamine:dolichol-phosphate N-acetylglucosamine-1-phosphate transferase in the liver, a enzyme which is specifically inhibited by the corynetoxins and other tunicaminyl-uracil antibiotics (Jago *et al.*, 1983; Cockrum *et al.*, 1989), are depressed.

Signs have appeared within four days to 12 weeks following introduction into a toxic paddock. Neurological disturbance may continue for up to 10 days after removal from toxic pasture with new cases appearing for the first two to three days.

ARGT has been experimentally reproduced in pigs (P.H. Berry and J.M. Howell, unpublished 1992) and a natural outbreak of tunicaminyluracil poisoning has also been recorded in this species (Bourke, 1987). Horses and a donkey have been affected in South Australia (R. Giesecke, pers. comm. 1992) and the former were also involved in the first recorded outbreak of floodplain staggers in New South Wales (E.O. Davis, pers. comm. 1991).

5.2. Laboratory Animals

Guinea pigs and two-week-old rats fed toxic annual ryegrass or dosed with extracts have shown tremor, ataxia, convulsions and death (McIntosh *et al.*, 1967; Berry *et al.*, 1976; Lanigan *et al.*, 1976; Peterson and Jago, 1977). Depression, weight loss and death have been observed in chickens (Lanigan *et al.*, 1976) as well as in adult rats and mice given similar material.

6. Pathology

6.1. The Natural Disease in Livestock

Only a small proportion of animals which die in field outbreaks of ARGT show gross pathological changes. In animals which do manifest lesions the liver is pale tan and enlarged (McIntosh *et al.*, 1967; Berry and Wise, 1975). Lungs may be congested and oedematous with frothy bronchial and tracheal contents. Further changes may be haemorrhages in epicardium, endocardium, skeletal muscle and alimentary tract; and sometimes free blood in the intestine.

Histopathological changes can be recognised in brains which have been removed and fixed immediately after slaughter of animals which have shown convulsions for several hours (Berry *et al.*, 1980*b*). Perivascular oedema, particularly in the cerebellar meninges is the consistent finding. Small haemorrhages, Purkinje cell necrosis and mononuclear cell infiltration of the cerebellar meninges may occasionally occur.

Diffuse vacuolar change of hepatocytes sometimes accompanied by small foci of neutro-phils is a common finding in the liver. In other tissues such as lung, kidney, intestine and lymph node, there may be small haemorrhages and oedema (McIntosh *et al.*, 1967; Berry and Wise, 1975).

6.2. Experimental Annual Ryegrass Toxicity

In experimental ARGT, whatever the dose rate or route of administration of corynetoxins, neurological signs are not observed before 40 hours (Berry and Vogel, 1982).

Findings from experimental studies of ARGT in nursling rats (Peterson and Jago, 1977), guinea pigs (Finnie and O'Shea, 1988, 1989), sheep (Jago and Culvenor, 1987; Berry *et al.*, 1980*a*) and cattle (Berry *et al.*, 1980*b*) suggest that neurological signs may be associated with a vasoconstrictor effect of the toxins. Evidence of restricted blood flow has been seen in the tail, hindlimbs and visceral tissues of two-week-old rats.

Histologically, lesions of focal necrosis consistent with anoxia occur throughout the brains of these animals. Sheep and cattle experimentally treated with toxic ryegrass or corynetoxins invariably show oedema of the central nervous system as seen in brains from naturally affected animals. Diffuse and focal degenerative changes have been observed in experimental sheep and cattle which survived for extended periods. The increased vascular permeability in the central nervous system of affected sheep and cattle may be associated with hypertension, secondary to the proposed vasospastic effect.

In sheep the lethal dose of the corynetoxin analogue tunicamycin, is about 35 µg/kg bodyweight when given subcutaneously and 3–5 mg/kg for corynetoxins administered orally as bacterial galls. The total lethal dose was of the same order whether given as a single dose or as repeated smaller doses, the maximum interval tested being nine weeks between doses (Jago and Culvenor, 1987).

The hepatotoxicity seen in the field has also been confirmed experimentally (Berry *et al.*, 1976; Berry *et al.*, 1982). Vacuolation of hepatocytes is again observed, but in addition the livers of experimental cases may also show individual hepatocyte necrosis, biliary hyperplasia, fatty change and hepatocytic regeneration (Berry *et al.*, 1982).

7. The Plant Disease

7.1. Recognition of Infected Annual Ryegrass in the Field

Although infected annual ryegrass has a patchy distribution in the field it can be detected for a short period each year, when galls are present in the developing inflorescences, between head emergence and when the pasture dries out (Price, 1973; Stynes and Wise, 1980). During this period, bright yellow bacterial slime can be seen on infected tillers. However, infection is more difficult to detect when the slime dries out during warm weather, or is present in reduced quantities due to a predominance of nematodes.

7.2. Collection of Ryegrass for Laboratory Examination

Laboratory examination of grain threshed from mature ryegrass is a more reliable method of detection. Ryegrass plants are collected by hand in areas of dense ryegrass, on clay rather than on sandy soil, and along water courses where infection levels have been shown to be highest. In random surveys, fields that have been cropped during the previous year are preferred for sampling because of the strong influence of cropping history on the level of infection (Stynes and Wise, 1980). Ryegrass seed is threshed from the plants and sufficient grass is usually collected to provide 50 g of seed.

7.3. Laboratory Examination of Ryegrass for the Presence of Anguina agrostis, Clavibacter toxicus *and the Corynetoxins*

Small samples (10 g) of threshed seed are rapidly screened by viewing over a light box where the outline of flask-shaped galls and oval-shaped seed show clearly through lemmas and paleas. Galls containing nematodes are darkly pigmented whereas toxic galls colonised by *C. toxicus* are usually yellow and less pigmented (Price, 1973; Stynes *et al.*, 1979). Nematode larvae can be identified after removal from galls hydrated on damp filter paper in a petri dish at 5°C overnight.

Larger samples are screened by flotation in ethanol [analytical reagent grade (AR), C_2H_5OH)] which has a specific gravity of 0.8. This allows galls (specific gravity about 0.3) and other light plant material to float while seeds (specific gravity about 1.0) and heavier plant debris sink. Samples are rapidly mixed with ethanol by stirring in suitable container and when the galls float to the surface, excess alcohol is added until they flow over for collection on a sieve. The sievings are washed in water, dried and examined on a light box. The number of galls containing nematodes and those colonised by bacteria are counted to provide estimates of the level of infection per gram of threshed grain. This method consistently detects single galls in samples of 50 g which contain about 75 000 seeds.

The South Australian Department of Agriculture provides a nematode/bacterium screening service for farmers wanting to know the toxicity status of paddocks.

Identification of the corynetoxins in toxic gall extracts involves high performance liquid chromatography (HPLC) (Cockrum and Edgar, 1985) and fast atom bombardment mass spectrometry (Cockrum *et al.*, 1989). These analyses may be obtained through the CSIRO Division of Animal Health, Parkville, Vic. 3052, Australia.

8. Diagnosis

Neurological signs and mortality in livestock grazing senescent annual ryegrass, blowaway grass or annual beard grass are suggestive of corynetoxin poisoning and related syndromes. Further indications are provided by elevated serum levels of liver-specific enzymes and detailed pathological examination (McIntosh *et al.*, 1967; Berry *et al.*, 1976; Berry *et al.*, 1980*b*; Berry *et al.*, 1982). The most definitive enzyme to assay for ARGT diagnosis is, however, uridine diphospho-N-acetylglucosamine:dolichol-phosphate N-acetylglucosamine-1-phosphate transferase which is specifically inhibited by the corynetoxins and other tunicaminyluracil antibiotics (Jago *et al.*, 1983; Cockrum *et al.*, 1989). The diagnosis of ARGT and related syndromes also depends on the demonstration of grass infected by *Anguina* spp. and *C. toxicus* in the diet of affected animals and ultimately requires the chemical identification of the toxins in the bacterially infected feed.

9. Treatment of Affected Animals

Treatment of affected livestock has usually proved unsuccessful. Convulsions associated with ARGT can be stopped with general anaesthetics and or the tranquiliser chlordiazepoxide (Richards *et al.*, 1979). However, use of this drug in natural outbreaks is severely restricted by the need for continued nursing and supportive therapy during sedation following dosing.

No natural immunity to ARGT has been demonstrated. A vaccine and toxin scavenging agents for treating livestock are being developed at the CSIRO Division of Animal Health, Parkville, Vic. 3052, Australia.

10. Agronomic Control

Control of ARGT is based on the combined use of a number of practices, aimed at eliminating ryegrass, breaking the nematode life cycle, or preventing the development of toxic galls. Pre and post emergence herbicides are used to control ryegrass in crops. Control in pasture depends on heavy grazing and the use of herbicides in the spring to reduce seed set and control gall formation, burning of pasture residues in summer to destroy galls and seed already formed, and scarification in autumn to encourage the germination of ryegrass followed by selective herbicidal control (Price, 1973; Stynes and Wise, 1980).

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