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From the Department of Biochemistry, Norwegian College of Veterinary Medicine, Oslo.

ALVELD-PRODUCING SAPONINS

II. TOXICOLOGICAL STUDIES

By

Signe Videm Abdelkader, Lovro Čeh, Inger W. Dishington and *Jens Gabriel Hauge*

VIDEM ABDELKADER, SIGNE, LOVRO ČEH, INGER W. DISHINGTON and JENS GABRIEL HAUGE: *Alveld-producing saponins II. Toxicological studies.* Acta vet. scand. 1984, *25*, 76—85. — The phototoxic lamb disease alveld, prevalent in South-Western Norway, is caused by ingestion of Narthecium ossifragum. Earlier studies have shown that peroral administration of large amounts of crude saponins from this plant elicits the disease. Such saponins have now been purified further by 2 different methods (A and B). Two A type preparations resulted in alveld when fed to 2 lambs. The most highly purified preparation (type B) did not cause alveld in the 2 lambs tested. Lambs vary, however, in their susceptibility to the disease. Both types of preparations led to increases in serum aspartate aminotransferase, bilirubin and 5′-nucleotidase in rats when injected intraperitoneally in amounts of 50 or 100 mg/kg body weight. Cannulation of the bile duct showed that injected saponins reduced both the volume of bile and the amounts of bilirubin and bile acids excreted. Histological changes seen in the light microscope were, except for the most peripheral parts of the liver, hardly noticable. These observations support the view that saponins are the liver-toxic agents responsible for alveld. The possibility is discussed that the effect arises through a change in the lipid environment of carrier-mediated transport systems.

alveld; saponins; toxicology.

Lambs grazing on pastures with a rich growth of Narthecium ossifragum often suffer from the phototoxic disease alveld. Photosensitization is elicited by the accumulation in the circulation of phylloerythrin, the excretion of which by the liver is retarded because of a toxic substance in the plant (*Ender* 1960, *Dishington & Laksesvela* 1976). N. ossifragum is rich in saponins, and their role as the causative agent in alveld was suggested by *Ender* (1955, 1960) on the basis of experiments with peroral administration of crude saponins from this plant. These saponins have recently been purified, and their structures studied (*Čeh &*

Hauge 1981). The dominating saponin, named narthecin, has a branched trisaccharide on C-3 of the steroid sapogenin, glucose and arabinose being attached to galactose. When isolated according to *Ender*, as done in the initial stage of the present work, the saponins undergo partial hydrolysis because of enzymes in the press juice, yielding a mixture with 3, 2, 1 and 0 carbohydrate molecules attached. With another method (*Čeh & Hauge* 1981) mainly saponins with intact trisaccharide were isolated and crystallized.

The aim of the present work was to study the effect of both of these types of saponin preparations on the liver function of lambs and rats.

MATERIALS AND METHODS

Saponin preparation

N. ossifragum leaves were obtained and saponins of type B, as well as furostanol, prepared as described earlier (*Čeh & Hauge* 1981). Saponin B consists largely of narthecin, with a small amount of the minor saponin xylosin and of desarabonarthecin (Fig. 2a in *Čeh & Hauge* 1981). Two versions of this product were used, both crystallized from 40 % ethanol (B1 and B2). The product B2 was further recrystallized several times and melted fairly sharply at 235°C. Their hemolytic indexes were as given in Table 1.

Table 1. Hemolytic index.

Preparation	ml/g
A1	80.000
A2	190.000
A3	>175.000
B1	180.000
B2	ca 200.000

Saponin A, a concentrate of intact narthecin, xylosin and partial hydrolysis products of these (Fig. 2b in *Čeh & Hauge* 1981) was prepared largely according to *Ender* (1955). The frozen leaves were stored, ground, pressed and the press juice left at room temperature for ca. 1 week. The saponin-containing precipitate which gradually appeared was extracted with boiling ethanol. After evaporation to dryness, chlorophyll and other lipophilic substances were extracted with ethyl ether. The product

at this stage is termed A1. After repeated reprecipitation from aquous ethanol, a product judged to be not less than 90 % saponin was obtained (saponin A2). Some remaining chlorophyll was removed with 0.2 NaOH and final crystallization from aquous ethanol yielded saponin A3.

The hemolytic index of these preparations was measured according to *Büchi & Dolder* (1950). It is equal to the volume in ml of a 2 % dilution of cow blood which is completely hemolyzed in 24 h by 1 g of saponin. The method was standardized with a commercial saponin. Because of low solubility in water, only approximate values were obtained for some preparations.

Clinical chemical determinations

Serum ASAT, ALAT, alkaline phosphatase (AP), γ-glutamyltransferase (γGT), bilirubin and BSP retention were measured as done routinely in our clinical laboratory. The 4 enzymes were measured according to recommendations by the Scandinavian Committee on Enzymes and using a Gemsaec centrifugal analyzer. Assays at 37° were also set up for serum leucinaminopeptidase (LAP) and 5′-nucleotidase (5′-NUC) according to published procedures. Bile bilirubin was determined as above, and bile acids assayed enzymatically with a commercial kit.

Feeding experiments with lambs

The lambs, of hemoglobin type BB, grazed on a fenced off pasture area without N. ossifragum and protection against sun light. A suspension of saponin in 1/4 l of 0.9 % NaCl was given by stomach tube twice a day. For 2 successive years, 2 twin lambs weighing approximately 20 kg were given saponin, and 2 other twin lambs on the same pasture served as controls.

Studies with more lambs would have been desirable. Production of pure N. ossifragum saponin for feeding to larger numbers of lambs was, however, not feasible. We turned instead to a small laboratory animal, the rat, to study the effect of these saponins on its liver function.

Feeding experiments with rats

Twelve male Wistar rats, weighing ca. 125 g at the start of the experiment were divided in 3 groups of 4 each. Group A received 60 mg saponin A1 per day, given by stomach tube, group B 40 mg saponin B1. Group C served as controls. The rats had

free access to standard feed pellets and water. BSP-retention, serum bilirubin and enzymes were measured at intervals in blood samples collected from one of the lateral tail veins (*Videm* 1980).

Intraperitoneal administration in rats

Male rats were given 50, 100 or 150 mg saponin per kg body wt. The saponin preparations were suspended in sterile 0.9 % NaCl so that the volume to be injected was ca. 1 ml. Control rats received NaCl only. Before injection and before blood sampling the rats were calmed by a subcutaneous injection of Hypnorm® Vet. (Mekos). Blood for analysis was sampled after 24 h. It was immediately centrifuged and the serum stored at —20°C for up to a week or at —80°C for longer storage time. The animals were euthanized with CO_2 after blood sampling and their bodies investigated for macroscopically visible changes. Pieces of liver from rats given 50 and 100 mg/kg and of control rats were prepared for light microscopic examination. The sections were stained by the hematoxylin-eosin method.

Bile secretion studies in rats

Rats were adapted to confinement cages by being placed in such cages for some hours daily for several weeks. After pretreatment with intraperitoneally injected saponin, and 0.9 % NaCl in the controls, polyethylene tubes were inserted by surgery in the stomach and in the bile duct. 0.9 % NaCl, 1.5 l/h, was pumped into the stomach and bile collected for 12 h.

RESULTS

Feeding experiments with lambs

In the first experiment 1 lamb was given saponin A1 in amounts gradually increasing from 7 to 28 g/day. After 12 days it showed clinical evidence of alveld. Increased BSP-retention and serum γGT levels were noted 6 days earlier. A second lamb was given saponin B1 in amounts increasing from 3 to 10 g/day. After 17 days, when the supply of B1 was exhausted, it showed no signs of alveld. Control lambs also remained healthy. Analysis of faeces from the lambs given saponin showed that full conversion to sapogenin had taken place.

The following year, 1 lamb was given saponin A2 in amounts increasing from 2 to 20 g/day. On day 16 alveld was diagnosed,

increases in γGT having been observed some days earlier. A second lamb was given saponin B2, increasing the dosage gradually from 5 to 15 g. After 23 days, when saponin B2 was exhausted, this lamb as well as the controls remained healthy.

Feeding experiments with rats

One group of rats was given saponin A1, another group saponin B1, as described under Materials and Methods. The experiment was terminated after 75 days. The total saponin dose administered per kg body wt was then larger than the total dose A1 giving alveld in the lamb. The rats receiving saponins showed reduced weight gain, 32 % lower for saponin A1, 23 % for B1. There was, however, no effect on serum bilirubin, BSP-retention or several serum enzymes with origin in the liver. Instead of peroral administration we chose then to study the effect of small amounts of saponin injected intraperitoneally.

Intraperitoneal administration in rats

Effects on liver function was observed for all doses, although variable and weak at the 50 mg level (Fig. 1). Results are shown only for ASAT, 5′-NUC and bilirubin. It became clear after some experiments that effects on ALAT were smaller but strongly correlated with those on ASAT, that AP showed much variation also in the controls, that γGT was absent from normal sera, and that changes in LAP were small even for serious liver dysfunction. These assays were therefore not continued. Experiments with 150 mg/kg were also discontinued, since the effects on the organism were too strong to serve as a model for the relatively weak liver effects in alveld.

Fig. 1 shows that saponins prepared by the 2 methods, A and B saponin, had similar effects. The A preparations, with their higher proportion of partial hydrolysis products, gave about the same response as the more homogenous B preparations. Pure narthecin had much the same effect as A2 and A3. When all carbohydrate was removed from the molecule, yielding sarsasapogenin, the 3 liver parameters were found to be in the normal range. Effect on the liver thus is dependent on sugars esterified to the C-3 hydroxyl. A few experiments were done with mono- and di-saccharides still attached, and with the furostanol form. The monosaccharide gave a very weak effect, even at the highest

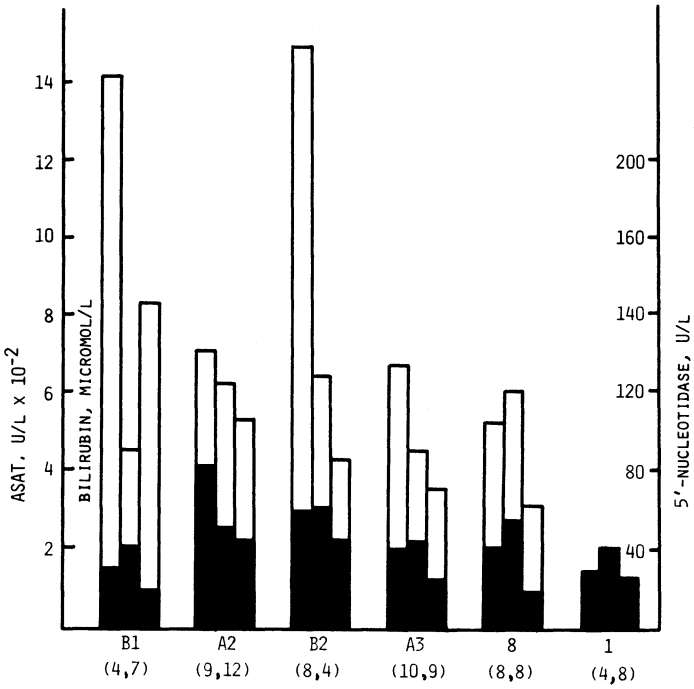

Figure 1. Effect of intraperitoneal injection of saponin preparations A2, A3, B1, B2, narthecin (8), and sarsasapogenin (1) on some serum components. ■ 50 mg/kg, □ 100 mg/kg. In each group of columns, the left is ASAT, the midde is bilirubin and the right is 5'-NUC. The columns represent mean values. The figures below the columns are numbers of rats on dose 50 and 100 mg/kg, respectively.

dosage level, whereas the disaccharides were more potent. Sugars in both end of the molecule, however, as in furostanol, making it water soluble, removed the effect on the liver.

Necropsy of the animals showed macroscopic changes varying with the dosage. For 50 mg/kg of a B-type saponin preparation, there were no easily observable macroscopic changes, except for the occurrance of ascites in a few cases. At 100 mg/kg, visible changes appeared in most cases. The changes consisted in some scattered fibrin threads at the liver surface and a tendency of pale discolouring of the superficial parts of the liver. Ascites was no constant finding, but had a higher incidence than at the lower dose level. When ascites was present, the fluid was clear and sometimes slightly yellow.

At a dose level of 50 mg/kg, there were virtually no histological changes that could be observed in the light microscope.

At the 100 mg/kg level, focal necrosis of the most peripheral liver tissue was observed. In these areas leucocytic infiltration was present. As for the main part of the liver, however, changes could hardly be noted.

Studies of bile secretion in rats

The results of an experiment in which saponin B2 was injected 12 h before the start of bile collection are shown in Table 2. There was a clear effect on the volume of bile secreted in 12 h for both 50 and 100 mg saponin per kg. The total amounts of bilirubin and bile acids secreted were significantly reduced only at the higher dosage level. Variation introduced by the operation may have obscured an effect at the 50 mg/kg level.

Table 2. Effect of saponin B2 on bile secretion.

Dose mg/kg	Rat no.	Volume ml	Bilirubin mmol	Bile acids mmol
0	1	12.5	0.250	0.230
	2	10.0	0.060	0.121
50	3	8.7	0.052	0.137
	4	8.0	0.464	0.087
	5	4.5	0.018	0.068
100	6	2.0	0.012	0.038
	7	2.0	0.042	0.037

DISCUSSION

Alveld was experimentally produced by feeding 2 saponin preparations of type A with hemolytic index 80,000 and 190,000. Two preparations of type B with index 180,000 and 200,000, produced by a different method, did not elicit the disease. It could thus be argued that alveld was caused not by saponins but by some uncharacterized minor component present in A-preparations but absent in B-preparations. The evidence obtained in the rat experiments suggest, however, that it is the saponin component which affects the liver function. No clear difference between A and B preparations were found in these studies. They affected the liver excretary function with similar efficiency, as shown by the concentration in serum of bilirubin and the enzyme 5′-NUC. 5′-NUC is an enzyme which like AP, LAP and γGT is bound to liver cell membranes, and which appears in the blood of patients

with cholestasis (*De Broe et al.* 1975). The lack of effect of the B preparations on the 2 lambs thus most likely is an expression of biological variation. Lambs with hemoglobin type BB are more susceptible than AA and AB lambs, but even for BB lambs on pastures rich in N. ossifragum almost half escape the disease (*Laksesvela et al.* 1977, *Laksesvela & Dishington* 1983).

The biological effects of saponins have been reviewed by *Cheeke* (1971). In addition to the hemolytic effect, possibly caused by reaction with cholesterol in the erythrocyte wall, effects have been observed on blood and liver cholesterol levels, growth, bloating of ruminants, smooth muscle activity and enzyme activity. *Coulson* (1957) found that as much as 2—3 % alfalfa saponin in the diets was necessary to produce growth depression in rats. In the present study approximately 0.3 % saponins in the dietary intake caused 20—30 % growth reduction. Saponins are poorly absorbed, accounting for their 10—1000 times higher toxicity when administered intravenously than when given orally (*Cheeke* 1971).

Effects on liver function of saponins given perorally or intraperitoneally have, to our knowledge, not been reported. Some substances having structural similarities with N. ossifragum saponins and other saponis are, however, known to be cholestatic. From the plant Lippia rehmanni has been isolated a substance named icterogenin, the structure of which is closely related to triterpenoid sapogenins (*Heikel et al.* 1961). Icterogenin, in doses of 1 to 4 g, introduced by stomach tube into the rumen of sheep caused temporary biliary retention without gross visible damage to liver cells. In experiments with rabbits, oral administration was ineffective. Intraperitoneal administration of 100—150 mg/kg, however, had marked effects on biliary excretion. This is the same dose level which gave good response to N. ossifragum saponins intraperitoneally in rats in the present study. Some hours after the administration of icterogenin the volume of the biliary excretion diminished markedly and the rate of elimination of coproporphyrin, phylloerythrin and bilirubin fell to very low levels. The most sensitive variable was the bile volume, as observed also in the present study.

The hepatic dysfunction in alveld is not accompanied by pathological changes visible in the light microscope (*Ender* 1955). Icterogenin and C-17-alkyl substituted androgens and estrogens likewise act without concomitant histological changes. (*Heikel et al.* 1961, *Zimmermann* 1974). The action of Narthecium sapo-

nins on the rat liver, given intraperioneally, appear to fall in the same category, changes being noticable only on the liver surface.

C-17-alkyl substituted androgens and estrogens cause intrahepatic biliary stasis (*Zimmermann* 1974). Ethinyl estradiol is one such compound, and for this information concerning its mechanism of action is available. *Davis et al.* (1978) have shown that administration of ethinyl estradiol to rats alters the composition and structure of surface membrane lipids and decreases hepatic Na^+, K^+-ATPase activity and bile flow 50 %. Treatment with a nonionic detergent restored membrane viscosity and lipids towards normal as well as Na^+, K^+-ATPase activity and bile flow. Strong correlation between bile flow and N^+, K^+-ATPase activity has been observed earlier (*Simon et al.* 1977). It is conceivable that icterogenin and N. ossifragum saponins likewise act through changing the lipid environment of the hepatocyte Na^+, K^+-ATPase and/or another membrane carrier involved in bile secretion.

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SAMMENDRAG

Alveldproduserende saponiner II. Toksikologiske studier.

Den fototoksiske lammesykdommen alveld, som forekommer særlig hyppig i Syd-Vest-Norge, skyldes inntak av Narthecium ossifragum. Tidligere studier har vist at peroral tilførsel av store mengder råsaponin fra denne planten fremkaller sykdommen. Slike saponiner er nå renset videre med to forskjellige metoder (A og B). To A-preparater resulterte i alveld da de ble gitt til to lam. De høyest rensede preparater (type B) forårsaket ikke alveld i de to lam som ble undersøkt. Lam varierer imidlertid i deres mottagelighet for sykdommen. Begge typer preparater førte til økning i serumkonsentrasjonene av aspartataminotransferase, bilirubin og 5′-nukleotidase når de ble injisert intraperitonealt i rotter i doser på 50 eller 100 mg/kg. Med gallefistel ble det vist at saponininjeksjon førte til reduksjon både i gallevolum og i mengde bilirubin og gallesyrer utskilt. Lysmikroskopiske histologiske forandringer kunne knapt påvises, bortsett fra i de mest perifere deler av leveren. Undersøkelsene støtter det syn at saponiner er det levertoksiske agens som forårsaker alveld. Den mulighet diskuteres at effekten oppstår gjennom en forandring i lipidomgivelsene for visse membrantransportbærere.

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Reprints may be requested from: J. G. Hauge, the Department of Biochemistry, Norwegian College of Veterinary Medicine, P. O. Box 8146 Dep, Oslo 1, Norway.