Hepatic protein variation among Romneys selected for resistance or susceptibility to sporidesmin-induced liver damage. A quantitative two-dimensional polyacrylamide gel electrophoresis study

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ABSTRACT

Quantitative two-dimensional polyacrylamide gel electrophoresis has been used to search for variation among liver proteins in flocks of sheep which had been selected for either resistance or susceptibility to sporidesmin-induced liver damage. The goal was identification of proteins whose DNA sequences might be used to select animals for resistance to facial eczema. The quantitative gel techniques were used because of their ability to detect variability among many proteins in a single tissue sample. Differences in relative proportion were tested for 62 proteins among 5 ewes from each of the resistant and susceptible flocks. There were 7 proteins which showed statistically significant differences ($P<0.05$) between the flocks. When an analysis was made of changes in the relative proportions of proteins 2 days after experimental challenge of resistant flock ewes (n=8) with sporidesmin, 0.13 mg per kg body weight, 25 of 79 liver proteins showed significant change ($P<0.05$). This included partial loss of a 25 kDa, pI 5.7, protein which was also three times more abundant in undosed susceptible than undosed resistant females. These results are discussed in terms of the possible relationships between protein variation and the magnitude of toxic tissue damage.

Keywords: Sheep; facial eczema; sporidesmin; proteins; quantitative 2D PAGE.

INTRODUCTION

Facial eczema is caused by the fungal metabolite sporidesmin which produces a photosensitizing disease secondary to damage to the liver and bile ducts. We have been using two-dimensional polyacrylamide gel electrophoresis (2D PAGE) to search for protein variation associated with sporidesmin-induced liver damage. 2D PAGE can be used to separate up to several thousand proteins from a single tissue sample (O'Farrell, 1975) so that differences between individuals and populations can be assessed. Our techniques combine the high resolution of 2D PAGE with the use of laser densitometry to measure the amount of each of the separated proteins. Our goal is identification of proteins which might affect the magnitude of sporidesmin-induced injury. We have chosen analysis of liver proteins because of the potential for this organ to modify sporidesmin toxicity, either through metabolism or binding of the toxic compound, and because of the role of sporidesmin-induced liver and biliary tract damage in producing many of the symptoms and consequences of facial eczema (Mortimer, 1963; Morris *et al.*, 1991). In this paper we report an analysis of differences among the liver proteins from ewes bred in flocks selected for either resistance or susceptibility to sporidesmin-induced liver damage and we examine the effect of sporidesmin dosing on liver protein composition.

MATERIALS AND METHODS

Animals

The flocks which were used in this study have been described by Morris *et al.* (1989). These flocks had been selected either for resistance (R) or susceptibility (S) to sporidesmin-induced liver damage, in genetic studies initiated in 1975. Two experiments were carried out. In the first experiment quantitative 2D PAGE was used to examine liver protein differences between groups of five 1-4 year old females from each of the R and S flocks. There was one set of twins in the susceptible group but all other animals were from separate sires and dams. The breeding values (Morris *et al.*, 1989) for these animals ranged from -0.81 to -1.03 for animals from the R flock, and from 0.43 to 0.59 for animals from the S flock. In the second experiment a study was made of the effect of sporidesmin dosing on the liver proteins of 8 females from the R flock (breeding values -0.56 to -0.87). The 8 animals were by 6 sires but had different dams. Originally the experiment was designed so that 8 females from each of the R and S flocks were to be analysed. A natural outbreak of facial eczema substantially affected the susceptible animals prior to dosing with sporidesmin so that results are only available for the effects of sporidesmin dosing on the liver proteins of ewes from the R flock. Liver biopsies were obtained from these animals at approximately 7 months of age. Three months later they were orally dosed with sporidesmin

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(0.13 mg per kg body weight) and after 2 days they were killed and samples of liver were removed for analysis.

Quantitative 2D PAGE

Liver proteins were separated by 2D PAGE of ^{14}C-labelled samples using techniques previously described by Loong *et al.* (1986). Briefly, this required labelling by reductive methylation with [^{14}C]formaldehyde in the presence of sodium cyanoborohydride, isoelectric focusing of 200-400 µg of liver homogenate protein in a polyacrylamide tube gel, followed by separation of the focused proteins on a polyacrylamide slab gel in the presence of sodium dodecyl sulphate (SDS). This produced a two-dimensional separation in which proteins were resolved on the basis of differences in isoelectric point (first dimension focusing) and molecular weight (second dimension separation in the presence of SDS). The separated ^{14}C-labelled proteins were detected by autoradiography. The procedures which were used in this study detected the approximately 200-300 most abundant proteins which had been separated in the isoelectric point range 3.5-8.5 and in the 15,000-100,000 molecular weight range. Little variation could be detected when replicate analyses of individual samples was carried out.

The autoradiograms were analysed using a Molecular Dynamics (Sunnyvale, CA, USA) laser densitometer. Quantitative analysis was carried out on the scanned images of the gels using Molecular Dynamics ImageQuant™ software, version 3.0. The amount of each protein was measured using the Image Quant™ software and for each individual protein the quantitative data were calculated after subtraction of a background density value for the region of that protein spot. We have shown that the densitometer values are proportional to protein loadings within the analytical range used (data not included).

Analysis of data

For each set of measurements of proteins from a 2D PAGE separation the measured quantity of each protein was expressed as a percentage (x) of the total. This was done to minimise variation due to differences in total protein loaded on different gels, and due to differences in autoradiographic exposure between gels. Relative proportions of individual proteins belong to a class of multivariate observations called compositional data. Statistical analysis of the data was therefore carried out using the log ratio transformation advocated by Aitchison (1986). Within this analysis the log ratio log[x/(100-x)] can be shown to be the sum of a fixed part and a collection of random errors which should have a normal distribution. This permits the valid application of the t-test for comparing independent pairs of samples and the analysis of variance for independent samples from more than two populations.

RESULTS

Comparison of resistant and susceptible animals (Experiment 1)

For this analysis, each of the abundant proteins on the gels was assigned a spot number which was consistently used for that protein on all of the gels prepared from the 10 animals. Visual inspection of the separated protein patterns from the liver biopsies of 5 R flock and 5 S flock ewes showed 32 proteins which appeared to vary in amount between individuals. Twenty of these proteins were either missing, or detected, in only 1-2 of the 10 animals, and 12 showed flock related differences in abundance (abundant or detectable in 4-5 animals from one flock and in only 1-2 animals from the other flock).

Quantitative analysis of protein variation, using densitometry of the gels, was carried out for 62 of the most abundant protein spots including the 32 protein spots which had been detected as varying in the visual inspection. Seven protein spots varied significantly ($P < 0.05$) between the R and S flocks (Table 1). There were greater proportions of two of these protein spots (19 and 97) in the susceptible flock and there were greater proportions of the other 5 in the resistant flock. Only 3 of the protein spots (20, 103, 175) had been detected as varying in the previous visual inspection of differences. Subsequent inspection of the gels revealed visually detectable flock related differences in proportion for spots 19 and 97, but not for spots 21 and 119 which showed smaller quantitative differences between the two flocks. The symmetrical shapes of 5 of the 7 spots were consistent with their being single proteins. Spots 19 and 21 however, although having symmetrical shapes, were close to other proteins so that it was difficult to quantify them.

TABLE 1: Proteins which showed significant ($P<0.05$) differences in percentage amount between ewes from the R and S flocks. Results are means and standard deviations for 5 animals in each group. The protein numbering system is discussed in the text.

Protein number	Approximate		Amount (% of the total quantified proteins)	
	isoelectric point	kilodaltons	R flock	S flock
19	5.7	25	0.3±0.4	1.3±0.7
20	5.5	23	0.7±0.3	0.2±0.1
21	5.6	24	0.8±0.3	0.3±0.2
97	6.5	31	0.2±0.2	1.0±0.3
103	6.3	19	0.9±0.3	0.1±0.2
119	6.8	39	0.7±0.2	0.3±0.1
175	7.4	22	0.4±0.2	0.1±0.1

Isoforms of proteins can generally be detected on 2D PAGE gels as a pair, or series, of spots separated in either the vertical (size) or, more usually, the horizontal (pI) dimension. Heterozygotes contain spots at both, or all, positions but homozygotes contain abundant spots at one, or a few, positions. None of the singificant variation detected in the present study appeared to be due to variation among isoforms.

Effect of dosing with sporidesmin (Experiment 2)

Visual analysis of the separated proteins from the pre-dose livers of the 8 R flock ewes showed many similarities to, and some differences from, the protein patterns of the 5 R flock ewes which had previously been analysed. Some of the differences may reflect progress with selection for facial eczema resistance as the animals in the R versus S flock comparison were born 2-4 years before the animals used in the dosing study.

The effect of sporidesmin dosing on liver proteins was analysed using a matched pair procedure in which differences

caused by dosing were independently examined for each animal. Visual inspection of the gels showed that a number of proteins changed in abundance after dosing with sporidesmin (Figure 1). Statistical analysis of change was carried out for 79 protein spots, including all proteins which showed obvious changes when the gels were visually inspected. There were 13 significant ($P < 0.05$) decreases and 12 significant ($P < 0.05$) increases in the relative proportion of protein spots after dosing. Nine of these protein spots showed more than 2 fold dose-related change (5 gains and 4 losses). Of the 7 proteins which had previously been shown to vary in a flock specific manner between R and S ewes (Table 1) only protein spot 19 showed a significant dose related change (mean 35% decrease after dosing).

FIGURE 1: Regions of 2D PAGE gels showing separation of liver proteins from a single Romney ewe before (top) and after (bottom) dosing with sporidesmin. Boxed region A shows 4 proteins whose abundance decreased, and boxed region B shows 2 proteins whose abundance increased, after dosing.

An attempt was also made to relate the magnitude of protein change to the relative degree of resistance or susceptibility of individual animals. This was difficult because of the restricted range of breeding values (-0.56 to -0.87), corresponding to only an approximately 12% difference in resistance (Morris *et al.*, 1989), between the extremes. Although no clear relationship was apparent the most susceptible animal sampled from the R flock (breeding value -0.56) showed the greatest, or least, change in percentage amount for 15 out of the 25 protein spots which varied significantly after dosing.

DISCUSSION

Differences among Romneys in their response to sporidesmin have been used to select lines which vary markedly in their resistance to facial eczema (Morris *et al.*, 1989, 1991). The biochemical and genetic basis of these differences is not known but some variability might be associated with differences among liver or biliary tract proteins. Some studies have indicated that natural resistance to sporidesmin-induced liver damage might be derived from differences in the hepatic microsomal drug metabolising system (Fairclough *et al.* 1978). Also as the liver and biliary system are major targets for sporidesmin action (Mortimer, 1963; Bhathal *et al.*, 1990), it was thought that there might be some variation between the liver proteins of facial eczema resistant and susceptible sheep.

In the first experiment, with 5 resistant and 5 susceptible Romney ewes, 7 out of 62 measured proteins varied significantly ($P<0.05$) between the two flocks. This result was itself significant ($P<0.05$) as only 3 differences due to random variation would be expected among 62 proteins. Some of these differences may therefore be due to random variability but some of the variation may reflect segregation of genes which affect resistance or susceptibility to facial eczema.

In the second experiment, dosing with sporidesmin apparently caused major changes in the profile of liver proteins. Some of these changes may be due to non-dose effects in the period between the pre-dose biopsy and dosing with sporidesmin. We have found similar changes however among the liver proteins of C57BL/6 mice experimentally exposed to sporidesmin (Lu and Jordan, in preparation). The post-dose protein measurements were made on liver collected 2 days after dosing the Romneys. Histological injury is minor at this time (Mortimer, 1963) so that changes due to inflammatory cell infiltration, necrosis and tissue repair should be minor. Altered protein expression should thus largely reflect both generalised responses of the liver to toxic challenge, and processes which are specific to the interactions of sporidesmin with target cells. It is therefore possible that some of the proteins which vary after sporidesmin dosing contribute to the magnitude of resistance or susceptibility to damage. To that extent we note that an approximately 25 kDa, pI 5.7, unidentified protein which was substantially more abundant in susceptible than resistant ewes also showed a partial decrease in expression after exposure to sporidesmin. The basis of this change is unknown however and it is possible that some of the other proteins which varied in this study may be more closely involved in the interactions of sporidesmin with the liver and biliary tract.

The value of the 2D PAGE approach is that searches for disease related protein associations can be made with few assumptions about the nature of the disease process. In addition the ability to obtain microsequence data from pro-

teins separated by 2D PAGE (Aebersold and Leavitt, 1990) offers the potential to design related DNA probes, which might be used for linkage studies and to identify genes and proteins which contribute to the disease phenotype.

CONCLUSIONS

We have demonstrated quantitative variation of liver proteins between Romney ewes bred in flocks selected for either resistance or susceptibility to facial eczema. We also detected protein variation which was caused by dosing with sporidesmin. Some of this variation may be related to processes which affect the extent of sporidesmin-induced liver injury. In this study we chose to make an initial analysis of protein variation in a small number of individual animals. We anticipate that in subsequent studies we will compare protein variation between a larger number of resistant and susceptible animals by comparing pools of samples from each of the resistant and susceptible flocks. We intend to use the results of this larger study to identify proteins for sequence analysis and design of DNA probes for use in selection of resistance, and for identification of the processes which contribute to resistance or susceptibility.

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