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Identification of autoantigens in canine autoimmune haemolytic anaemia

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SUMMARY

Autoantigens in canine autoimmune haemolytic anaemia (AIHA) were identified by immunoprecipitation using autoantibody eluted from the erythrocytes of affected dogs. At least three patterns of precipitated antigen were identified in six cases of AIHA. The most commonly precipitated antigen pattern was a combination of 42-kD and 29-kD peptides, associated with up to three other membrane components. These autoantigens may be canine glycophorins, which are of similar molecular mass, or may possibly represent an equivalent of the human Rhesus complex. An autoantigen identical in molecular mass to band 3, the erythrocyte anion channel protein, was precipitated in one case of AIHA, and unknown peptides of 37 kD and 100 kD were isolated by autoantibody from another dog. In one case, no antigens were precipitated by the eluted antibody, indicating that the autoantibody may have bound a non-protein membrane component such as phospholipid. Overall it is considered that the different patterns observed may reflect differences in the aetiology of the condition. In other studies, sera from dogs with AIHA failed to immunoprecipitate autoantigens, but were shown by immunoblotting to contain autoantibodies to proteins of the erythrocyte cytoskeleton. Such autoantibodies were also demonstrated in normal canine sera and it is suggested that they are unlikely to play a role in the pathogenesis of AIHA, but may be part of a normal clearance mechanism for damaged red blood cells.

Keywords erythrocyte autoantigens immunoprecipitation autoimmune haemolytic anaemia canine diseases

INTRODUCTION

Autoimmune haemolytic anaemia (AHIA) was one of the first diseases shown to have an autoimmune pathology, and has subsequently been described in humans and in domestic animals (Dodds, 1977). AIHA in dogs is a relatively common form of anaemia and provides an excellent model for the human condition, since the disease is clinically similar in both species (Halliwell & Gorman, 1989). Furthermore, the dog as a companion animal is exposed to many of the same environmental influences as humans. The disorder is characterized by a reduced erythrocyte lifespan *in vivo* and the presence of antibodies directed against autologous erythrocytes (Packman & Leddy, 1990). Although the mechanisms by which autoantibody-coated erythrocytes are destroyed in the spleen, or by complement-mediated lysis, are well documented (reviewed by Sokol & Hewitt, 1985), little is known about the underlying aetiology of the disease.

In the dog, numerous diagnostic tests have been prescribed for the detection of erythrocyte autoantibodies (Jones & Darke, 1975; Campbell & George, 1984; Jones *et al.*, 1987, 1990), but the identity of the autoantigens to which these antibodies bind is unknown. The aim of the current work was therefore to identify the erythrocyte autoantigens in cases of canine AIHA, since this would be a logical first step towards understanding the aetiology of the condition. In particular we wished to determine whether the same, or different antigens were involved in each case, since any variation might reflect differences in the aetiology of the disease.

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MATERIALS AND METHODS

Animals

Eight dogs with AIHA were admitted to the department of Veterinary Medicine at the University of Bristol. The diagnosis was based on clinical signs, a positive papain test (Jones & Darke, 1975) and a positive direct enzyme-linked anti-globulin test (DELAT) (Jones *et al.*, 1987). Details of the cases are summarized in Table 1.

Blood samples were obtained by cephalic venepuncture and taken into citrate anticoagulant. Greyhound blood donors maintained at the same establishment, or healthy dogs admitted for routine neutering, were used as a source of normal blood.

Table 1. Case details of dogs with autoimmune haemolytic anaemia

Case no.	Breed	Age (years)	Sex	Haemoglobin (g/dl) (normal > 11·9)	Prednisolone therapy	Concurrent disease
1	Springer spaniel	2·5	F	2·9	–	–
2	Doberman pinscher	4	F*	14·0	+	–
3	Jack Russell terrier	8	M	3·4	–	–
4	Irish setter	8	F	9·4	+	–
5	Doberman pinscher	7	F*	12·9	+	–
6	Doberman pinscher	3	F*	13·8	+	Thrombocytopenia
7						
Sample A	Springer spaniel	6	F	6·9	–	Bronchitis
Sample B				13·1	+	
8						
Sample A	Afghan hound	3	F	11·2	–	Thrombocytopenia
Sample B				17·9	+	

* Neutered.

Direct enzyme-linked anti-globulin test
Erythrocyte-bound IgG autoantibody was detected using a modification of the DELAT (Jones *et al.*, 1987). Washed erythrocytes were glutaraldehyde fixed as described and 50 μl of a 2% suspension of the cells added to duplicate wells in round-bottomed microtitre plates (Nunc). The erythrocytes in each well were incubated successively with 50-μl volumes of rabbit antiserum to canine IgG Fc (Nordic) diluted 1/500 and alkaline-phosphatase-conjugated goat antibody to rabbit IgG (Sigma) diluted 1/1000. Antisera were diluted in phospate-buffered saline (PBS) pH 7·4 containing 0·2% w/v bovine serum albumin (BSA); after each incubation, which lasted for 1 h at 37°C, the cells were washed three times in diluent. Finally, 100 μl of substrate solution containing 1 mg/ml p-nitrophenyl phosphate disodium hexahydrate (Sigma) in a pH 9·6 carbonate buffer were incubated in each well for 1 h at 37°C. After pelleting of the erythrocytes by centrifugation, 50 μl of each supernatant were transferred into the wells of fresh, flat-bottomed microtitre plates (Dynatech) and the absorbance measured at 405 nm using an automated plate reader (Titertek Multiskan).

Elution of autoantibody from erythrocytes
Autoantibody was eluted from erythrocytes by ether treatment (Rubin, 1963) as described by Petz & Garratty (1980). Eluates were stored at −20°C and, after thawing, any precipitated material was removed by centrifugation.

Erythrocyte membrane preparations
Erythrocyte ghosts (Dodge, Mitchell & Hanahan, 1963) were prepared by hypotonic lysis of washed erythrocytes as described by Day *et al.* (1989). Cytoskeletal spectrin was obtained by incubating erythrocyte ghosts for 20 min at 37°C in a 0·3 mM phosphate buffer, pH 7·6 (Ungewickell & Gratzer, 1978). After centrifugation at 30 000 *g* for 1 h, the spectrin-containing supernatant was collected and concentrated.

The protein concentration of each preparation was determined by the method of Bradford (1976).

ELISA for eluted autoantibody
Erythrocyte eluates were assayed for autoantibodies by an ELISA, using erythrocyte membranes pooled from six normal dogs as the target antigen. A 25 μg/ml suspension of erythrocyte ghosts was sonicated on ice for 30 sec at 4 μM peak-to-peak amplitude in an MSE sonicator. Flat-bottomed microtitre plates (Dynatech) were coated with membranes by drying down the sonicated ghost suspension at 37°C overnight. The coated plates were stored at 4°C and used within 4 months. After non-specific binding sites on the plates had been blocked with PBS–1% BSA overnight at 4°C, duplicate wells were incubated with eluate samples. This was followed by successive incubations with 1/1000 dilutions of rabbit antiserum to dog IgG Fc (Nordic) and alkaline-phosphatase-conjugated goat antibody to rabbit IgG (Sigma). Finally, substrate solution prepared as for the DELAT was incubated in each well and the absorbance measured at 405 nm. All volumes were 50 μl/well, antisera were made up in blocking buffer and the plates were washed three times with PBS containing 0·5% v/v Tween 20 after each incubation, which lasted for 1 h at 37°C.

Immunoprecipitation of erythrocyte autoantigens
Canine erythrocyte autoantigens were immunoprecipitated using a modification of the technique described by Laing *et al.* (1987). Washed erythrocytes were pooled from four or five normal dogs and biotin labelled as described. Aliquots (0·25 ml) of packed, labelled erythrocytes were rotated for 1 h either at room temperature or at 37°C with 3–5 ml of autoantibody-containing eluate or autoimmune serum, respectively. Samples of each eluate were retained for ELISA both before and after incubation with erythrocytes. After washing three times in PBS, the cells were incubated with 1 ml rabbit antiserum to dog IgG Fc (Nordic) diluted 1/500 in PBS and previously absorbed with normal canine erythrocytes. The labelled erythrocytes were further washed three times and erythrocyte ghosts were prepared from each sample by hypotonic lysis. These membranes were dissolved in 6 ml PBS containing 2% v/v Triton X-100 (PBS-Triton) and insoluble cytoskeletal elements were removed by centrifugation at 30 000 *g* for 30 min at 4°C. Proteolysis was inhibited by the addition of PMSF to a final concentration of 2 mM and the immune complexes in each detergent extract were immobilized by rotation with 50 μl of a 50% suspension of sepharose protein A (Sigma) for 1 h at room temperature. The sepharose beads were washed six times in PBS-Triton and the

Table 2. Autoantibody levels bound to erythrocytes and free in erythrocyte eluates from dogs with autoimmune haemolytic anaemia

Case no.	Erythrocyte-bound IgG (DELAT OD)	Erythrocyte autoantibody in eluate (ELISA OD)		Autoantigens immunoprecipitated
		Before absorption	Absorbed with fresh erythrocytes	
1	0·475	0·756	0·062	+
2	0·389	0·063	0·037	+
3	0·694	0·960	0·038	+
4	0·286	0·178	0·030	+
5	0·930*	0·484	0·022	+
6	0·506	0·087	0·035	+
7				
Sample A	0·414	0·061	0·035	−
Sample B	0·213	0·036	0·041	−
8				
Sample A	0·193	0·193	0·088	−
Sample B	0·218	0·121	0·072	−
Normal dogs†	0·071	0·030	0·034	−
(s.d.)	(0·011)	(0·007)	(0·009)	

*DELAT run using rabbit anti-dog IgG whole molecule (Sigma).
†$n=4$.

immune complexes eluted in 50 μl SDS electrophoresis buffer containing 8 M urea and 5% v/v 2-mercaptoethanol.

SDS–PAGE

SDS–PAGE was performed in 12% polyacrylamide gels according to the method of Laemmli (1970), using the BioRad Mini Protean II. Apparent molecular masses were calculated from migration relative to standard protein markers (Sigma SDS-6H).

Where necessary, gels were developed with Coomassie brilliant blue, silver or periodic acid–Schiff (PAS) stains.

Western blotting

Proteins separated by SDS–PAGE were transferred to nitrocellulose by Western blotting (Towbin, Staehelin & Gordon, 1979; Burnette, 1981), using a semi-dry apparatus (LKB) with a continuous buffer system as described by the manufacturer.

Detection of biotin-labelled proteins after blotting

When biotin-labelled immunoprecipitates were analysed, blots were blocked in PBS–5% BSA overnight at 4°C and incubated for 1 h at 37°C with peroxidase-conjugated extravidin (Sigma) diluted 1/500 in blocking buffer. After washing six times in PBS–0·05% Tween, blots were developed by luminography (Laing, 1986), which is a method akin to autoradiography.

Immunoblotting

Erythrocyte autoantigens recognized by serum antibodies were detected by immunoblotting. Erythrocyte membrane preparations pooled from six normal dogs were subjected to SDS–PAGE at 4 μg protein/lane and blotted on to nitrocellulose as described above. Blots were blocked in PBS–5% BSA–0·1% Tween overnight at 4°C and cut into strips corresponding to individual lanes. The nitrocellulose strips were incubated successively with canine serum diluted 1/50 and a 1/500 dilution of peroxidase-conjugated sheep antiserum to dog IgG (a gift from Mr D. Patel). All sera were made up in blocking buffer and each incubation lasted for 1 h with continuous agitation at 37°C, followed by six washes in PBS–0·05% Tween. Finally, strips were developed by luminography (Laing, 1986).

RESULTS

Detection of erythrocyte autoantibody

Blood samples were obtained from eight dogs with AIHA and the levels of erythrocyte-bound IgG autoantibody assayed. In each of these cases, high levels of bound IgG were demonstrated using the DELAT (Table 2). Bound immunoglobulin was eluted from the erythrocytes by ether treatment and its specificity tested by measuring its capacity to bind canine erythrocyte membranes in an ELISA, both before and after absorption with canine erythrocytes. The results are summarized in Table 2. It can be seen that in all cases except one (case 7, sample B), erythrocyte membrane-reactive IgG, which was removed by absorption with fresh erythrocytes, was detected in the eluate. The data also show that there is no simple relationship between the DELAT reading for erythrocyte-bound IgG and the ELISA measurement of eluted antibody. In particular, erythrocyte eluates from case 7 consistently gave low or negligible ELISA readings despite the presence of large amounts of erythrocyte-bound IgG detected by the DELAT. It should be noted that typical eluates contained less than 5 μg/ml IgG, at which concentration there was no non-specific binding in the ELISA (data not shown).

By contrast with AIHA cases, samples obtained from normal dogs gave low DELAT readings for erythrocyte-bound IgG, with no demonstrable autoantibody in the corresponding eluate.

Immunoprecipitation using eluted autoantibody

Erythrocyte eluates from the eight cases of AIHA were tested for their ability to immunoprecipitate canine RBC antigens.

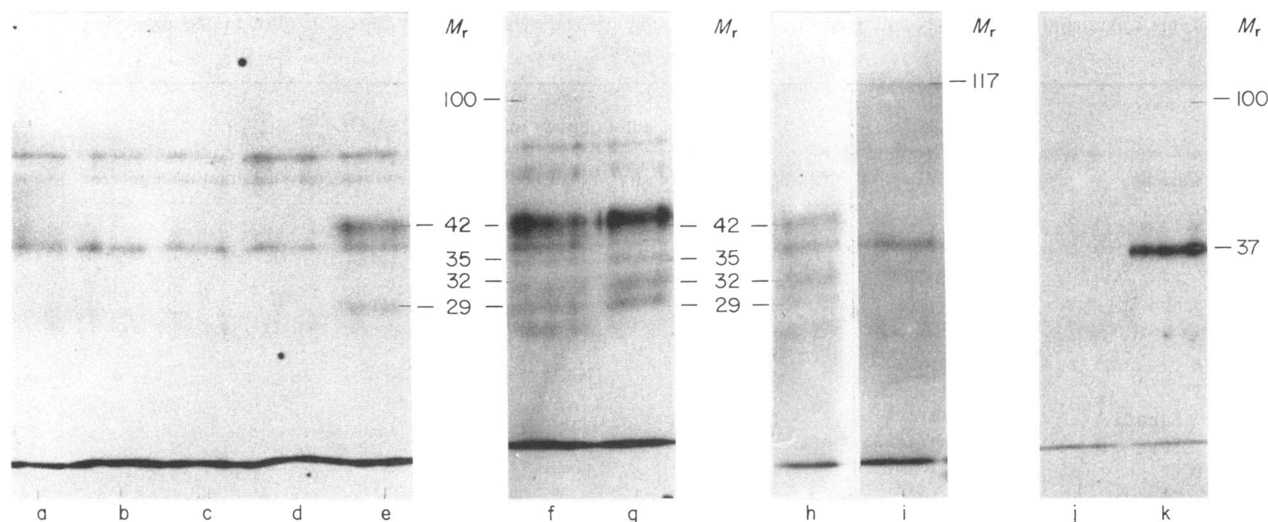

Fig. 1. Immunoprecipitation of biotin labelled canine erythrocyte membranes by autoantibody eluates derived from AIHA-positive dogs. Immunoprecipitates were analysed by SDS–PAGE, blotted on to nitrocellulose and developed by luminography. Biotin-labelled erythrocytes were incubated with eluates from cases of AIHA as follows: a, PBS (negative control); b, normal dog; c, AIHA case 7; d, AIHA case 8; e, AIHA case 1; f, AIHA case 2; g, AIHA case 3; h, AIHA case 4; i, AIHA case 5; j, normal dog; k, AIHA case 6. The apparent molecular mass of the specific bands precipitated is indicated in kD. Lanes a–e, f+g, h+i and j+k are from four different blots.

Table 3. Apparent molecular mass of specific bands immunoprecipitated by erythrocyte eluates from dogs with autoimmune haemolytic anaemia

	Band apparent molecular mass (kD)						
Case no.	117	100	42	37	35	32	29
1	−	−	+	−	−	−	+
2	−	+	+	−	+	+	+
3	−	−	+	−	+	+	+
4	−	−	+	−	−	+	+
5	+	−	−	−	−	−	−
6	−	+	−	+	−	−	−

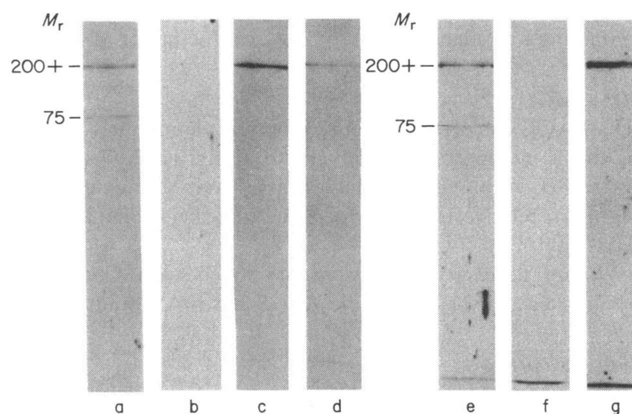

Fig. 2. Immunoblotting of canine erythrocyte membranes probed with sera from AIHA-positive or normal dogs. Blotted membranes were incubated with sera as follows, and developed by luminography: a, normal dog 1; b, normal dog 2; c, normal dog 3; d, normal dog 3 (serum previously absorbed with 200 μg canine erythrocyte ghosts and 100 μg canine spectrin for 1 h at 37°C); e, AIHA case 1; f, AIHA case 2; g, AIHA case 3. The apparent molecular mass of the bands detected by immunoblotting is indicated in kD. Lanes a, b, c+d and e–g are from four different blots.

Figure 1 shows that, when eluates from cases 1–6 were tested, one or more specific bands luminesced after SDS–PAGE analysis of the immunoprecipitates, blotting and development (lanes e–i and k). Four additional bands of apparent molecular mass 62, 53, 37 and 27 kD, which could be seen with varying intensity in each track, were judged to be non-specific since they were also present in control precipitates prepared using either PBS in place of the eluate (lane a), or an eluate from normal dog erythrocytes (lane b). The 37-kD band which was precipitated by the eluate from case 6 appeared to be specific, since it was considerably more intense than the non-specific band of similar molecular mass seen in a control lane from the same gel, which had been prepared using a normal dog erythrocyte eluate (compare lanes j and k).

Eluates from cases 7 and 8, and from normal dogs, failed to precipitate any specific bands (lanes c, d and b). This negative result for cases 7 and 8 was repeated using the second sample from each dog (result not shown). In case 7 this failure may be attributed to the low or negligible titre of eluted autoantibody (Table 2), but this does not account for the negative result for case 8 where the corresponding ELISA values for eluates were relatively high.

The apparent molecular masses of the antigens precipitated using erythrocyte eluates derived from each AIHA-positive dog are summarized in Table 3. There appear to be at least three distinct patterns in individual cases. The combination of a 42-kD and a 29-kD antigen, together with up to three other peptides, was the most frequent pattern seen. A single antigen of 117 kD, and a 37-kD band together with a 100-kD peptide, were each isolated in one case.

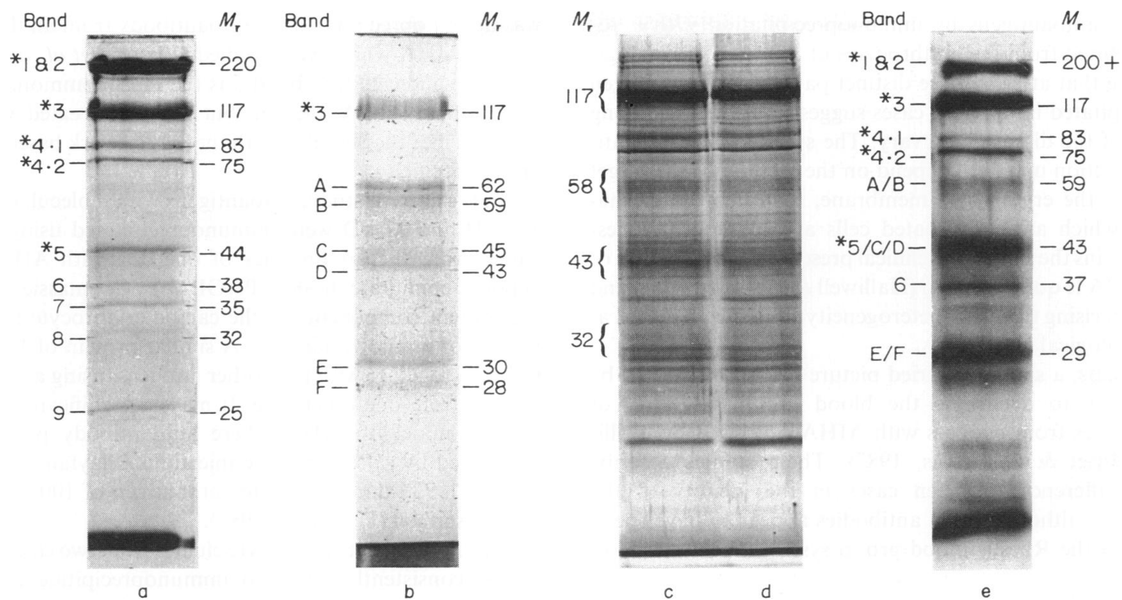

Fig. 3. SDS-PAGE analysis of canine erythrocyte membranes. a, Coomassie blue protein stain (20 μg protein/track); b, PAS glycoprotein stain (30 μg protein/track); c and d, silver protein stain (2 μg protein/track). Glycoprotein, which is stained orange in the original, is rendered grey here and marked with a bracket. In lane c the membranes had been biotin labelled, in lane d the membranes were unlabelled; lane e, biotin-labelled membranes blotted on to nitrocellulose and developed by luminography (2 μg protein/track).
* Bands labelled according to the nomenclature for human erythrocytes (Fairbanks *et al.*, 1971); other bands have no obvious equivalent in humans and have been given arbitrary designations. The apparent molecular mass of the major bands is indicated in kD. lanes a, b, c+d and e are from four different gels.

In contrast to the results obtained using erythrocyte eluates as a source of autoantibody, autoimmune sera from the eight cases of AIHA failed to immunoprecipitate any canine erythrocyte antigens (results not shown).

Immunoblotting

Sera from the eight AIHA-positive dogs were also used to probe immunoblots of canine erythrocyte membranes (Fig. 2). In each case, and when normal sera were tested, a band with an apparent molecular mass of over 200 kD was revealed by luminescent development. This band was taken to represent a true autoantigen, since its intensity varied with different sera (compare lanes b and c; f and g) and was markedly reduced if the serum had been previously incubated with canine erythrocye membrane components (compare lanes c and d). A weaker band of 75 kD was sometimes detected when sera from AIHA-positive and normal dogs were tested (lanes a and e), with further faint bands of 83 kD and 44 kD revealed after longer exposure times (results not shown). The development of these weaker bands was found to be poorly reproducible in repeat experiments.

SDS–PAGE erythrocyte membranes

In order to characterize the protein and glycoprotein components of canine erythrocyte membranes, erythrocyte ghosts were prepared and analysed by SDS–PAGE. Gels were stained with Coomassie brilliant blue, silver or PAS stains and the results are shown in Fig. 3. Nine major protein bands, ranging in molecular mass from 220 to 25 kD were revealed using Coomassie blue (lane a). Of these, the five with the highest molecular mass could be labelled according to the nomenclature adopted for human erythrocytes (Fairbanks, Steck & Wallach, 1971; Steck, 1974), while the smaller proteins appear to have no obvious counterpart in humans (Barker, 1991). The greater sensitivity of the silver stain enabled many additional minor protein bands to be visualized (lane d).

Several glycoproteins in the canine erythrocyte membrane were identified using the PAS and silver stains (lanes b and d). Canine band 3 was shown to be heavily glycosylated by both stains, which also revealed a further three diffuse glycoprotein bands not identified by Coomassie blue. Using the PAS stain, each of these three diffuse areas could be resolved into two distinct bands.

Biotin-labelled erythrocytes were shown to be suitable for immunoprecipitation, since most of the major protein and glycoprotein membrane components were clearly identifiable after SDS–PAGE, blotting and luminescent development (lane e). Furthermore, labelling did not affect the apparent molecular masses of these components (compare lanes c and d).

Some of the autoantigens isolated in this study migrate closely in SDS–PAGE with major protein and glycoprotein staining components of the erythrocyte membrane. Thus, the band of over 200 kD which was identified by sera after immunoblotting corresponds with bands 1 and 2, and the 117-kD band immunoprecipitated by an erythrocyte eluate from case 5 correlates with canine band 3. Also, the 100-kD band precipitated by eluates from cases 2 and 6, and the 37-kD band precipitated by an eluate from case 6, each comigrate with unidentified peptides which stain with Coomassie blue. Finally, the 42-kD band precipitated by four eluates corresponds in SDS–PAGE with glycoprotein band D, and the associated antigens of 32 and 29 kD migrate closely with glycoproteins E and F respectively.

DISCUSSION

The current work describes the identification of seven canine

erythrocyte autoantigens by immunoprecipitation with autoantibody eluted from the erythrocytes of AIHA-positive dogs. The finding that at least three distinct patterns of autoantigen were precipitated in different cases suggest that the underlying aetiology of the disease may vary. The stimulus for autoantibody production may well depend on the identity of the target antigens on the erythrocyte membrane, although the mechanisms by which antibody-coated cells are subsequently destroyed remains the same. The clinical presentation and course of canine AIHA is quite variable (Halliwell & Gorman, 1989) and it is not surprising that such heterogeneity should be reflected at an immunological level.

In humans, a similarly varied picture has been revealed by investigations to determine the blood group specificity of autoantibodies from patients with AIHA (reviewed by Mollison, Engelfriet & Contreras, 1987). These studies describe apparent differences between cases in the identity of the autoantigens, although often antibodies appear to have specificity within the Rhesus blood group system (Weiner & Vos, 1963).

The close SDS–PAGE migration of the 42-kD and 29-kD autoantigens, which were precipitated by antibody from four dogs, with major PAS staining bands from the erythrocyte membrane suggests that these antigens may be canine glycophorins. Glycophorins are heavily sialated erythrocyte membrane glycoproteins which bear blood group antigens in man, including those of the MN and Ss systems (Hamaguchi & Cleve, 1972). Antibody from occasional cases of human AIHA has been reported to show specificity for N (Bowman *et al.*, 1974; Dube *et al.*, 1975; Cohen *et al.*, 1979) and S (Johnson *et al.*, 1978). The other peptides of 32, 35 and 100 kD which were variably precipitated with the 42 kD and 29 kD antigens in the present study may represent minor related glycophorins, such as those recently described in the rat (Laing *et al.*, 1988). However, where multiple membrane components are isolated by immunoprecipitation it must be remembered that the technique can allow the precipitation of 'innocent bystander' proteins that are non-covalently associated with the true antigen.

Alternatively, it might be suggested that the 42 kD and 29 kD autoantigens precipitated here represent not glycophorins, but a canine equivalent of the human Rhesus antigen. The RhD antigen is associated with membrane components of 28–33 kD (Gahmberg, 1982; Moore, Woodrow & McClelland, 1982) and 45–100 kD (Moore & Green, 1987), and human AIHA autoantibodies often appear to have specificity within the Rhesus system (Weiner & Vos, 1963). Although no Rhesus equivalent has been described in the dog, the complex appears to play an important role in maintaining erythrocyte viability (Ridgewell *et al.*, 1983) and so might be expected to be conserved between species.

Autoantibody eluted from one case of canine AIHA in the current series immunoprecipitated an antigen of 117 kD, which corresponded with band 3 in SDS–PAGE migration. Band 3 is the major erythrocyte membrane glycoprotein (Fairbanks *et al.*, 1971), functions as the erythrocyte anion channel (Cabantchik, Knauf & Rothstein, 1978) and in humans bears ABH blood group determinants (Karhi & Gahmberg, 1980; Finne, 1980). Only in rare cases of human AIHA has band 3 been implicated as an autoantigen, by the demonstration of autoantibodies with specificity for A (Szymanski, Roberts & Rosenfield, 1976; Parker *et al.*, 1978). Surprisingly, in a recent report, band 3 alone was immunoprecipitated by autoantibody from all the cases of human AIHA which were studied (Victoria *et al.*, 1990). Such data may indicate that band 3 is the most common, if not the only human AIHA antigen, but must be treated with some caution since they conflict with the earlier work based on blood group specificity.

In the current study, autoantigens with molecular masses of 100 kD and 37 kD were immunoprecipitated using antibody eluted from the erythrocytes of one dog with AIHA. These peptides comigrate in SDS–PAGE with Coomassie blue staining protein components of the canine erythrocyte membrane, but are of unknown identity. A similar protein of 100 kD was isolated, together with four other antigens, using autoantibody derived from one other case. It may be significant that in the murine model of AIHA where autoantibody production is stimulated by rat erythrocyte injections (Playfair & Marshall-Clarke, 1973), an erythrocyte autoantigen of 100 kD has also been described (Day *et al.*, 1989).

The finding that erythrocyte eluates from two cases of canine AIHA consistently failed to immunoprecipitate erythrocyte membrane components calls for some comment. This negative result may be explained in one case by the apparently poor recovery of autoantibody after elution from erythrocytes. In contrast, eluates from the second dog contained relatively high levels of autoantibody, suggesting that the antigens in this case may be non-protein components of the erythrocyte membrane similar to the phospholipid antigens described in humans (Hazeltine *et al.*, 1988) and in the mouse (Cox & Hardy, 1985; Kawaguchi, 1987; Hardy & Cox, 1990). Alternatively, the negative result may be due to incomplete biotin labelling of the erythrocyte membrane, although this procedure was shown to be very effective.

In a survey of only eight cases, it is not surprising that there was no correlation between the success or pattern of autoantigen precipitation (Tables 2 and 3), and the breed, age or sex of affected animals (Table 1). Also, the use of corticosteroid treatment in some cases precludes comparison with the severity of the anaemia. However, it is noteworthy that the results of autoantibody elution and immunoprecipitation were more variable in cases of AIHA where concurrent disease was present, again suggesting differences in aetiology.

The use of immunoblotting revealed that sera from both normal and AIHA-positive dogs contained autoantibodies that react with erythrocyte components. Judging from their apparent molecular mass, the autoantigens involved were identified as bands 1 and 2, which comprise spectrin, the major protein of the erythrocyte cytoskeleton (Bennet, 1985). Autoantibodies to such internal components cannot bind to intact erythrocytes and so would not be expected to be pathogenic. Rather, the presence of these autoantibodies in normal canine sera suggests that they may play a beneficial role in the immune clearance of damaged erythrocytes from the circulation. Naturally occurring autoantibodies to the erythrocyte cytoskeleton in normal humans have also been reported (Lutz & Wipf, 1982; Ballas, 1989), and Day *et al.* (1989) showed that mice can be stimulated to produce anti-spectrin autoantibodies by the injection of autologous erythrocyte membranes.

The failure of sera from AIHA-positive dogs to immunoprecipitate the erythrocyte autoantigens, identified using corresponding erythrocyte eluates, may be due to low titres of autoantibody in the serum samples. Erythrocyte autoantibody

can be detected in such sera (Jones & Darke, 1975; Jones, 1986), but in AIHA most of the pathogenic antibody is probably absorbed onto the erythrocytes. Alternatively, autoantibody remaining in the serum may be of lower affinity or directed against cryptic, non-protein or hidden epitopes which cannot be precipitated from intact erythrocytes.

Canine sera also failed to immunoprecipitate the cytoskeletal autoantigens identified by immunoblotting. This negative result was to be expected, since not only are the internal proteins of intact erythrocytes inaccessible to the autoantibodies in immunoprecipitation, but the cytoskeleton remains insoluble after detergent extraction (Maddy, 1982) and is removed by centrifugation.

The results of the current work reveal a fascinating paradox of autoimmunity: autoantibodies to certain erythrocyte membrane components are rarely produced and may have potentially lethal effects, whilst antibodies to adjacent proteins are normal, benign and possibly advantageous.

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