Uveitis, vitreous humour, and klebsiella.

I. Binding studies with rabbit antisera

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SUMMARY Uveitis occurs in a proportion of patients with ankylosing spondylitis, and an increased faecal isolation of the Gram-negative micro-organism *Klebsiella pneumoniae* has been reported from such patients. Immunological cross-reactivity between *K. pneumoniae* and bovine vitreous humour has been studied by 2 different antibody binding techniques: ^{125}I -labelled antigen binding assay with and without carrier, and beta-galactosidase enzyme-immunoassay. Sera from rabbits immunised with whole klebsiella micro-organisms or klebsiella extracts were found to bind labelled vitreous humour antigens to a greater extent ($p < 0.001$) than sera from rabbits immunised with *Escherichia coli*, *Streptococcus pyogenes*, and $\phi\text{X 174}$ virus or sera from the same rabbits before immunisation. It is suggested klebsiella micro-organisms may carry antigenic determinants which resemble vitreous humour antigens.

In patients suffering from ankylosing spondylitis (AS) acute anterior uveitis (AAU) is a frequent peripheral complication, occurring in approximately 20 to 30% of cases.¹ The Gram-negative micro-organism *Klebsiella pneumoniae* has been isolated more frequently from faecal cultures of AS patients during active phases of the disease than during inactive phases, and the presence of uveitis was considered to be a good indicator of disease activity.² *K. pneumoniae* was isolated in 13 out of 17 episodes (76%) in AS patients with uveitis, compared with an overall klebsiella isolation rate of 30% in AS patients without uveitis.³ Similar results have been reported by Eastmond and coworkers,⁴ though another group could not confirm these observations.⁵

Over 90% of AS patients carry the HLA-B27 antigen, while it is present in only 8% of a Caucasian population.⁶⁻⁸ In idiopathic acute anterior uveitis over 50% of patients carry HLA-B27,⁹ but, when AS occurs together with AAU, Geraint James found in a survey of 50 patients that the frequency of the B27 antigen rises to 98%.¹⁰ It would appear that HLA B27 provides a genetic link between AS and uveitis. Since klebsiella micro-organisms can not only be isolated from patients having AS and AAU, but also show cross-reactivity with some HLA B27 lymphocytes,¹¹ it is possible that the

underlying pathological mechanism in these 2 diseases may be similar.¹²

In this study we have examined the immunological similarities between klebsiella extracts and bovine vitreous humour.

Materials and methods

PREPARATION OF VITREOUS HUMOUR

Crude vitreous humour (CVH) from a bovine source (cow or calf) was obtained by posterior incision of thawed eyeballs. This material was heavily pigmented and probably contained fragments from the retinal and uveal layers of the eye. CVH was homogenised in a Potter-Elvehjem homogeniser (6 strokes), then cooled on ice, and sonicated with an MSE 25 sonicator at medium setting and 8 μM peak-to-peak amplitude. The resultant material was centrifuged in a bench centrifuge for 10 minutes at room temperature to remove particulate matter. The slightly opaque, yellow tinged supernatant solution was removed and stored at 4°C or dialysed against distilled water and lyophilised.

PREPARATION OF BACTERIAL ANTIGENS

Klebsiella pneumoniae (MX 100) was obtained from a faecal culture of an AS patient (Middlesex Hospital, patient 100), subcultured, then grown on MacConkey or nutrient agar plates and harvested in phosphate buffered saline (PBS). After 2 washes with the same buffer the cells were suspended in PBS for treatment with ultraviolet irradiation, ethanol, or formalin

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Table 1 Rabbit antisera used in vitreous humour binding studies. Rabbit antisera were prepared by either multiple site (MS) subcutaneous (SC), intravenous (IV), intraperitoneal (IP) injections of bacterial or viral antigens, with or without adjuvant

Rabbit	Antigen	Quantity of antigen	Adjuvant	Method of immunisation	Immunoglobulin content (mg/ml)
A	CVH (cow)	1 ml	IF	SC (MS)	11.3
B	<i>K.pneumoniae</i> (UV irradiated)	10 ⁸ organisms	CF	SC (MS)	17.0
C	<i>K.pneumoniae</i> (ethanol killed)	1.5 × 10 ⁸ organisms	—	IV	14.7
D	<i>K.pneumoniae</i> (formalin killed)	1.5 × 10 ⁸ organisms	—	IP	ND
E	KSP	1 mg	IF	SC (MS)	15.1
F	<i>E.coli</i> (UV irradiated)	10 ⁸ organisms	CF	SC (MS)	22.3
G	<i>S.pyogenes</i>	2 × 10 ⁸ organisms	—	SC (MS)	15.8
H	<i>Ent. aerogenes</i> (UV irradiated)	10 ⁸ organisms	CF	SC (MS)	ND
I	φX 174	1 ml	CF	SC (MS)	23.0

IF=incomplete Freund's. CF= complete Freund's adjuvant. ND= not determined.

prior to injection into rabbits. 'Klebsiella sonicate preparation' (KSP) as well as the origin of the micro-organisms used has been described elsewhere.¹¹ *Escherichia coli* (ML 30), *Enterobacter aerogenes* (NCTC 10066), and beta-haemolytic *Streptococcus pyogenes* type III (QEC D10) were prepared as above from MacConkey or blood agar cultures, and bacteriophage φX 174 was obtained commercially (10¹³ plaque forming units/ml, Miles Laboratories).

PREPARATION OF RABBIT ANTISERA

Rabbit antisera against the described antigens were obtained after varied regimens of immunisation, and the details are summarised in Table 1. New Zealand white rabbits 1.5–3 kg in weight were used, and each rabbit was immunised several times over a period of 3 to 6 months. All sera were divided into aliquots (0.2–0.5 ml) in plastic tubes and stored at –15°C until use, when the sera were thawed at room temperature and used immediately. Each antiserum was tested for its ability to agglutinate the homologous micro-organism (Widal or haemagglutination) or to bind the radiolabelled homologous antigen preparation. All sera used had strong reactions with their homologous antigens. The immunoglobulin content of the sera was determined after precipitation with 50% saturated ammonium sulphate and extensive dialysis against PBS. The protein content was estimated from $E_{280}^{1\%} = 12$, with lyophilised preparations (Table 1). Immunoglobulin content of preimmunisation sera ranged from 9.3 to 15.2 mg/ml (mean ± SD = 11.3 ± 2 mg/ml).

Donkey antirabbit IgG serum (Wellcome Reagents Ltd.) was titrated to assess the equivalence point

with several sera obtained from immunised and unimmunised rabbits. It was found that with donkey antirabbit to rabbit serum ratios of 16/1 to 4.5/1, with rabbit serum at 1/100 dilution, a broad plateau was obtained between serum ratios of 10/1 and 5.5/1. Therefore for all assays a ratio of 8/1 donkey antirabbit IgG serum to rabbit serum was used.

PREPARATION OF RADIOACTIVELY LABELLED VITREOUS HUMOUR

Crude vitreous humour (0.5 ml) was labelled with Na¹²⁵I (1 mCi; Radiochemical Centre, Amersham) by the method of Greenwood *et al.*¹³ The reaction mixture was dialysed against PBS containing 0.015 M sodium azide (PBS-A) and potassium iodide (0.01 M) in a small dialysis chamber with continuously running buffer. Portions of the labelled CVH (CVH-¹²⁵I) were chromatographed on Sepharose 6B (Pharmacia, London). A 2 × 18 cm column of gel was equilibrated with PBS-A containing 0.4% bovine serum albumin (BSA), and the column was developed with the same buffer (Fig. 1). In preliminary tests with rabbit anti-CVH serum fraction 1 alone showed substantial binding and was therefore used in all subsequent assays. This radiolabelled fraction had to be used within 4–6 days of preparation, and BSA (0.4% w/v) was included in all stages of testing.

RADIOLABELLED ANTIGEN BINDING ASSAY

CVH-¹²⁵I fraction 1 was diluted to approximately 4 × 10⁵ cpm per ml in assay buffer (PBS-A-0.4% BSA), then 50 μl of the antigen, and 50 μl of buffer

Fig. 1 Fractionation of ^{125}I -labelled crude vitreous humour (CVH), on a Sepharose-6B column; 10 μl aliquots were used to measure radioactivity. Only the first fraction was used in radiobinding assays.

was incubated with 50 μl of rabbit serum dilutions in plastic tubes (LP3-Luckhams) at 37°C for 30 minutes. The tubes were then allowed to cool to room temperature, and 50 μl of donkey anti-rabbit IgG serum at suitable dilutions was added to the tubes and incubation continued at 4°C overnight for 18-20 hours. 1.0 ml of PBS-A buffer at 1-2°C was added to the tubes and the precipitate separated by centrifugation at 4°C for 30 minutes at 3000 rpm in an MSE Mistral 6L centrifuge. 1 ml aliquots from the supernatants were discarded and the washing procedure repeated. The radioactivity in the tubes was counted in a Packard model 578 autoscintillation spectrometer (efficiency 69%). Four replicates per assay point were set up as well as tubes containing labelled antigen and buffer only, which were not washed, and radiolabelled antigen and buffer with donkey antirabbit serum, which were washed, to assess the total applied radioactivity and the blank loads respectively. The amount of radioactivity left in the test sample was expressed as the percentage of the total counts applied, and this was calculated from the formula:

$$\text{Percentage bound (\%B)} = \left(\frac{\text{Test} - \text{Blank}}{\text{Total} - \text{Blank}} \right) \times 100$$

RADIOLABELLED ANTIGEN CARRIER ASSAY

Radiolabelled antigen and rabbit sera were diluted as above and incubated at 37°C for 30 minutes. After the tubes had been cooled to room temperature 50 μl donkey antirabbit IgG serum at a dilution of 1/10 containing preimmunisation serum from rabbit A at 1/100 dilution, acting as carrier, was added, and the tubes were then incubated, the precipitates washed, and counted as above.

PREPARATION OF ENZYME LABELLED VITREOUS HUMOUR

Beta-D-galactosidase was conjugated to CVH by the method of Exley and Abuknesha.¹⁴ To 1 ml of solution containing 40 mg per ml of CVH in PBS, pH 7.1, was added 1 ml of buffer containing extensively purified beta-D-galactosidase (9 mg per ml) obtained from *E. coli* K12 (strain 3300), and 150 μl of 25% glutaraldehyde solution. The reaction mixture was stirred for 4 hours at room temperature and overnight at 4°C, then dialysed against 0.1 M phosphate buffer, pH 7.1, containing 0.1 M NaCl. It was then chromatographed on a Sephadex G25 (medium) column (1.5 $\times$ 40 cm) and the conjugate partially purified by affinity chromatography on a column of CH-Sepharose-4B-galactosylamine (Fig. 2). After washing with 0.01 M phosphate buffer at pH 7.0 the adsorbed conjugate was eluted with 0.05 M NaCl in the same buffer and fractions containing active enzyme, concentrated, and dialysed against PBS-A.

Fig. 2 Affinity chromatography of conjugate mixture on CH-Sepharose-beta-D-galactosylamine column. Protein (—) was followed by measuring absorbance at 280 nm and enzyme activity (---) at 405 nm.

Fig. 3 Vitreous humour- ^{125}I binding curves for various rabbit antisera (vitreous humour = A, *Klebsiella* SP = E, *Klebsiella* "whole organisms" = B, *E. coli* = F, ϕ X 174 = I) using coprecipitation method. Dashed lines (— — —) represent binding by sera obtained from I and A rabbits before immunisation, while binding curves for remaining preimmunisation sera fell between these 2 curves.

The enzyme activity was measured by the method of Craven and coworkers.¹⁵ Briefly, 200 μl of substrate solution of O-nitrophenyl-D-galactopyranoside (Sigma Ltd., London) at a concentration of 1 mg per ml reagent, in 0.1 M phosphate buffer, pH 7.0, containing 0.1 M NaCl, 0.01 M MgCl_2 , 0.01 M 2-mercaptoethanol, was added to enzyme containing solutions and the tubes incubated for 30 minutes at 30°C. The reaction was stopped by the addition of 1 ml of 1M Na_2CO_3 and the absorbance measured at 405 nm.

ENZYME LABELLED VITREOUS HUMOUR BINDING ASSAY

Aliquots of 50 μl of assay buffer and 50 μl of enzyme labelled CVH solution. In increasing concentrations were incubated with antiklebsiella serum (rabbit B, Table 1) at a single dilution for 1 hour at 37°C, then, after addition of 50 μl of donkey antirabbit-IgG

serum, overnight at 4°C. After the washing procedure similar to the one described for radiobinding assay the remaining enzyme activity in the precipitates was determined and expressed as bound activity.

Results

RADIOBINDING ASSAYS

The ability of various rabbit sera to bind CVH- ^{125}I antigen preparations is shown in Fig. 3 and 4. In each case the binding by test serum is compared with binding by serum obtained from the same rabbit before immunisation. The anti-KSP serum (E) bound to the antigen better than antiklebsiella micro-organisms serum (B) (Fig. 3). Anti-KSP serum binding levels compared with preimmunisation serum binding levels from the same rabbit (E) are highly significant at all dilutions (1/800 dilution, $t = 11.2$, $p < 0.001$), whereas those of serum B are

Fig. 4 Vitreous humour- ^{125}I binding curves for rabbit antisera (*Klebsiella* 2 = C, *Klebsiella* 3 = D, *Streptococcus pyogenes* = G) by a coprecipitation method. Hatched area represents range of binding curves for 5 sera obtained from 5 rabbits prior to immunisation (mean $\pm$ SD). Binding curve for antiserum to *Enterobacter aerogenes* (H) fell within the shaded area.

significantly raised only at lower dilutions. Rabbit E had been immunised with a concentrated extract of klebsiella antigens, and this may account for its greater binding of CVH-¹²⁵I compared with that of serum obtained from rabbit B. The *E. coli* antiserum (F) had a similar low vitreous humour binding capacity as serum B but antiphage ϕ X 174 serum (rabbit I) did not show any significant binding to CVH-¹²⁵I preparation. When sera from rabbits C and D (antiklebsiella) were tested (Fig. 4), their

avidities for CVH-¹²⁵I were only marginally better than that of antistreptococcus serum (G). It is possible that inactivating klebsiella micro-organisms with chemical reagents may cause an alteration of some of the surface antigens, thereby producing a loss in the degree of cross-reactivity with CVH-¹²⁵I. The anti-*Enterobacter aerogenes* serum (H) did not bind the CVH-¹²⁵I antigen.

The effects of adjuvant used during immunisation on the binding properties of the antisera would appear to have been negligible, since antienterobacter serum (H) did not bind CVH-¹²⁵I above levels obtained for preimmunisation serum, and despite the high immunoglobulin content of anti- ϕ X 174 serum (I) the binding was only marginally higher above the level of preimmunisation serum (Fig. 3).

The binding by sera B, E, and F may be due to the presence of cross-reactive IgG antibodies evoked by immunisation with Gram-negative antigens. This is particularly the case with anti-KSP serum (E), which was tested up to a dilution of 1/4000 in a binding assay with carrier (Fig. 5a) and to a dilution of 1/3000 without carrier (Fig. 5b).

Differences in the binding capacities of vitreous humour preparations from cow (Fig. 5a and 5b) and calf (Fig. 6) eyeballs were rather interesting. Rabbit A had been immunised with vitreous humour from cow eyeballs and did not bind calf-CVH-¹²⁵I (Fig. 6) with the same potency as cow-CVH-¹²⁵I (Fig. 5a and 5b), and there was a corresponding reduction in binding by anti-KSP serum (E) (Fig. 6).

Fig. 5 Cow vitreous humour-¹²⁵I binding curves of sera from rabbits immunised with cow vitreous humour (A) and klebsiella sonicate preparation (E) measured with (Fig. 5a) and without (Fig. 5b) added carrier. Since preimmunisation serum at low dilution is already present in each test with added carrier (5a), results are expressed as difference in binding between immunised and unimmunised sera from each rabbit. In assays without added carrier (5b) preimmunisation sera obtained from both rabbits gave almost identical binding curves.

ENZYME CONJUGATE BINDING ASSAY

Enzyme immunoassay (Fig. 7) showed significant enzyme-vitreous humour conjugate binding in the serum obtained from the rabbit immunised with *Klebsiella pneumoniae* micro-organisms (B) compared to serum obtained from the same rabbit before immunisation ($t=5.88$, $p<0.001$). Control estimations with unconjugated enzyme showed no difference in binding between klebsiella antiserum and control serum obtained from the same rabbit before immunisation.

The enzyme labelled CVH conjugate proved to be a more stable preparation than the radiolabelled vitreous humour, but the disadvantages of the technique were the extensive purification required in the enzyme preparation and the time-consuming double affinity chromatography necessary to obtain a useful reagent.

Discussion

Antisera raised against ultraviolet-light-inactivated klebsiella micro-organisms or against klebsiella sonicate extract have been shown to bind to a high

Fig. 6 Calf vitreous humour- ^{125}I radiobinding assay of antisera from cow vitreous humour (VH) (rabbit A) (—) and *klebsiella sonicate* (KSP) (rabbit E) (---) immunised rabbits (■). Preimmunisation sera (●) obtained from both rabbits gave almost identical binding curves.

molecular weight fraction from radiolabelled cow and calf vitreous humour preparations, by both radiobinding dilution assay and radiobinding carrier assay. A similar result of increased binding by a *klebsiella* antiserum was obtained with a galactosidase labelled vitreous humour conjugate used in an enzyme immunoassay. Sera obtained from rabbits immunised with chemically inactivated *klebsiella* micro-organisms or *E.coli* showed only a small increase in binding to labelled vitreous humour preparations.

One possible explanation for these results is that immunisation with Gram-negative bacteria such as *klebsiella* may evoke the synthesis of antibodies that bind to some antigens present within the bovine

eyeball. Partial structural similarity with vitreous humour could be due to the presence of carbohydrate components found in the cell walls and capsules of Gram-negative bacteria, since both substances appear to have a high carbohydrate content. The difference in binding of antiklebsiella sonicate serum between cow and calf vitreous humour antigens could have been due to the slightly different methods of preparations of these extracts or to the age and strains of animals, which came from separate sources. However, cow-CVH antiserum also showed decreased binding to labelled calf-vitreous humour, and this may be due to antigenic differences in the different vitreous humour preparations. The effect of coprecipitating serum in

Fig. 7 Binding activity of preimmunisation serum (control) and *klebsiella* antiserum, obtained from rabbit B, with galactosidase-vitreous humour conjugate (—) and unconjugated enzyme (---).

radiobinding assays was carefully assessed to avoid artefacts due to this source. Assay blanks with radiolabelled tracer and coprecipitating serum were tested over the whole dilution range, and the effect was found to be negligible. Variations in total immunoglobulins were also found not to affect the results, since antisera with the highest immunoglobulin levels, such as those raised against *E.coli* bacterium or ϕ X 174 virus, had little or no vitreous humour binding activity respectively.

Cross-reactivity between bacterial and mammalian antigens has been described by many authors. Antigenic cross-reaction between murine host and various species of salmonella micro-organisms has been proposed as an important factor in susceptibility to bacterial infection.¹⁶ Autoantibodies to gut tissue have been described following immunisation of rabbits with Gram-negative bacteria.¹⁷ Immunological cross-reactivity between group A streptococci and cardiac tissue has been suggested as a possible factor in the development of rheumatic fever, though the actual pathogenic mechanism remains uncertain.^{18, 19} Some Gram-negative bacteria share cross-reactive antigens with human tissues, and isoantibodies against blood group A and B substances can be demonstrated in man after ingestion of *E.coli* micro-organisms.²⁰

The studies reported here tend to suggest that antigens in Gram-negative bacteria may have immunological similarity not only to human blood group antigens but also to bovine vitreous humour, and this may be relevant to the observed increased isolation of faecal klebsiella in AS patients with uveitis.^{3, 4} The association of HLA B27 with AS and uveitis is now considered to be due not to a linked gene but to the B27 gene itself.²¹ One possible mechanism mediating such association is cross-reactivity with antigens coded by the major histocompatibility complex.¹² In mice cross-reactivity between H-2 alloantigens and synthetic²² as well as bacterial antigens²³ has been suggested as a possible explanation for low immune responses, thereby facilitating infection and producing an increased liability to disease. In man cross-reactivity between HLA and bacterial lipopolysaccharides has been described,²⁴ and recently we reported studies suggesting antigen similarity between HLA B27 and klebsiella, because monospecific B27 tissue typing sera were found to bind klebsiella antigens to a greater extent than non-B27 tissue typing sera.¹¹

Whether there is a direct connection between uveal antigens, HLA B27, and klebsiella is at the moment unclear, but antibody could be considered as a possible mediating link, since vitreous humour binding capacity is increased after immunisation with klebsiella antigens. The distinction between

uveal and vitreous humour antigens is not easy when dealing with antigenic preparations from closely related biological sources. Antiuveal antibodies have been reported in patients with uveitis and not found in control subjects, but the immunological origin of these antibodies was not studied.²⁵ These investigations and the results reported here would indicate that sera from AS patients with uveitis may be studied with uveal, vitreous humour, and klebsiella antigens.

In this study we have only suggested a possible immunological link between klebsiella and bovine eye antigens. In the following paper²⁶ direct competition studies have been carried out between klebsiella antigens and bovine eyeball extracts for specific antivitreous humour antibodies in an endeavour to demonstrate direct cross-reactivity.

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