

Uveitis, vitreous humour, and klebsiella.

II. Cross-reactivity studies with radioimmunoassay

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SUMMARY Radioimmunoassay with calf and cow vitreous humour-¹²⁵I and rabbit antivitreous humour serum has been employed to investigate the immunological cross-reactivity of vitreous humour with bacterial and mammalian tissue antigens. *Klebsiella* ultrasonic preparation at a dose of 10 000 µg/ml was found to inhibit the binding of vitreous humour by 25-100% ($p < 0.001$), compared with an inhibition of 5-30% by a similar quantity of *E. coli* ultrasonic preparation. Equivalent amounts of *Streptococcus pyogenes* antigen, bovine haemoglobin, and hyaluronic acid had no inhibitory effect, while horse spleen ferritin was found to inhibit vitreous humour binding between 0 and 10%. These results indicate that *klebsiella* micro-organisms have antigens which partially resemble some eyeball components. It is suggested that acute anterior uveitis of ankylosing spondylitis may be produced by anti-Gram-negative bacterial antibodies binding to cross-reacting eye antigens.

Acute anterior uveitis (AAU) or iridocyclitis is an inflammatory condition of the uveal tract which may occur in association with a number of arthritic disorders including ankylosing spondylitis (AS), Reiter's syndrome, and Still's disease. The reason for this association is unknown. However, an increased frequency of the histocompatibility allele HLA B27 has been found in these diseases.¹⁻³ *Klebsiella pneumoniae* has been isolated from faecal cultures of patients with active AS⁴ as well as from AS patients with uveitis.^{5,6} *K. pneumoniae* has been found to partially cross-react with antigenic determinants located on HLA B27 lymphocytes,^{7,8} and some of these could involve the HLA B27 antigen itself,⁹ or a closely associated gene product.¹⁰

In an endeavour to determine a possible pathological connection between Gram-negative micro-organisms and uveitis the structural properties of *klebsiella* micro-organisms have been investigated by competitive inhibition in a vitreous humour radioimmunoassay with bacterial and mammalian tissue antigen extracts.

Materials and methods

PREPARATION OF VITREOUS HUMOUR ANTISERUM

A New Zealand white rabbit was immunised with crude cow-vitreous-humour (CVH) in incomplete

Freund's adjuvant several times over a period of 6 months as described in the previous paper.¹¹ After 9 immunisations the rabbit was bled, the serum was separated and precipitated with half saturated ammonium sulphate, and dialysed against phosphate buffered saline (PBS), and the resulting antiovine vitreous humour immunoglobulin (anti-VH-Ig) preparation (A1) was stored at -15°C until use. A2 immunoglobulin preparation was obtained after 11 immunisations. Preparations were restored to original volume with PBS.

PREPARATION OF BACTERIAL ANTIGENS

Klebsiella pneumoniae (MX100) was isolated from a faecal culture of an ankylosing spondylitis patient and the bacterial extract 'klebsiella sonicate preparation' (KSP) was obtained as previously described.⁹ Briefly, *klebsiella* micro-organisms were grown on MacConkey culture plates, harvested, washed twice with cold PBS (4°C), resuspended (20 ml; 1.3×10^{13} micro-organisms), ultrasonicated, and centrifuged. Supernatant was filtered through Sephadex G25, dialysed against distilled water, and lyophilised. 100 mg residues were recovered which dissolved readily in PBS and $E_{1\text{cm}}^{1\%}$ at 280 nm was 11.8. A similar bacterial extract was prepared from *Escherichia coli* (ML 30) micro-organisms, and streptococcus group A antigen was obtained commercially (Difco).

MAMMALIAN TISSUE ANTIGENS

Mammalian tissue antigens used as controls in the comparative studies were (i) hyaluronic acid prepared

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from human umbilical cord (British Drug Houses); (ii) haemoglobin obtained from beef blood (Sigma); (iii) ferritin extracted from horse spleen (Miles Laboratories).

PREPARATION OF VITREOUS HUMOUR

Bovine vitreous humour was homogenised in a Potter-Elvehjem homogeniser and centrifuged to remove any large particulate matter. The vitreous humour was then ultrasonicated in an MSE 25 ultrasonicator, at medium setting, with 10-second bursts for 4 cycles, recentrifuged, and supernatant dialysed continuously against 10 litres of deionised water before being lyophilised. Calf vitreous humour obtained from younger animals was treated in a similar manner, but, after ultrasonication, the CVH was centrifuged in an MSE 18 centrifuge for 1 hour at 12 000 rpm at 4°C and then dialysed overnight at 4°C against 10 litres of PBS containing 0.1% sodium azide (PBS-A). The CVH was then desalted on fine Sephadex G25 (Pharmacia) before being lyophilised.

IODINATION AND FRACTIONATION OF VITREOUS HUMOUR

Crude bovine vitreous humour (CVH) (2 mg) was radiolabelled with 1 mCi of Na¹²⁵I (Radiochemical Centre, Amersham) as described in the previous paper.¹¹ After dialysis the ¹²⁵I-labelled vitreous humour was applied to a Sepharose 6B column (Pharmacia), equilibrated with 0.15 M PBS, pH 7.2, containing sodium azide (PBS-A), and the first fraction was collected as described,¹¹ stored at 4°C, and used in subsequent assays.

RADIOBINDING ASSAY

The assay buffer used in all experiments was 0.15 M PBS-A containing 0.4% bovine serum albumin (BSA) (Radioimmunoassay grade, Sigma). To 50 µl assay buffer in an LP3 tube (Luckhams) was added 50 µl of tracer CVH-¹²⁵I followed by 50 µl of rabbit anti-VH-Ig in serial dilutions. After 30 minutes' incubation at 37°C, 50 µl of coprecipitating donkey antirabbit immunoglobulin G serum (Wellcome Reagents) was added. The optimum dilution of second antibody was determined by measuring the quantity of first antibody precipitated by varying dilutions of coprecipitating antibody. Maximum precipitation was obtained with a dilution of 1/180 of coprecipitating donkey antirabbit IgG serum per 10 µg of anti-VH-Ig. The tubes were then left overnight at 4°C, washed twice with PBS-A containing 0.01 M potassium iodide, centrifuged at 3000 rpm for 30 minutes, and the radioactivity in the precipitate counted in a well-type gamma counter (Packard Model 578). Each serum dilution assay was carried

out in quadruplicate and percentage bound (%B) was expressed as:

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

SELECTION OF ANTISERUM DILUTION FOR RADIOIMMUNOASSAY

The dilution of antiserum that binds a given percentage of labelled antigen for use in the radioimmunoassay (RIA) was selected from binding curves, described in the previous paper,¹¹ and was taken as the dilution giving maximum difference in percentage bound of CVH-¹²⁵I, when comparing antivitreous humour serum with preimmunisation serum from the same rabbit. For instance, for a particular batch of vitreous humour, at a dilution of 1/2000 of anti-VH-Ig there was 30% binding of CVH-¹²⁵I compared with less than 1% binding by preimmunisation serum immunoglobulin, and therefore in this particular assay system percentage bound in absence of competitor (B₀) equals 30% (Fig. 1). For each new batch of labelled vitreous humour a radio-binding assay was carried out to determine the dilution point of maximum difference in binding between pre- and postimmunisation sera preparations for use in RIA.

RADIOIMMUNOASSAY

In the radioimmunoassay the concentrations of antibody and radiolabelled tracer remain constant, and increasing amounts of competitor are added to displace the label. A standard competition or inhibition curve is obtained with unlabelled CVH and compared with inhibition produced by equal quantities of other antigens. To 50 µl aliquots of CVH-¹²⁵I, diluted as for radiobinding assay, was added 50 µl of buffer containing increasing concentrations of competitor, then 50 µl of anti-VH-Ig at the dilution determined by the radiobinding assay and incubated at 37°C for 30 minutes. After cooling, 50 µl of coprecipitating serum was added, and the tubes were left overnight at 4°C. The washing procedure was carried out as for the radiobinding assay, radioactivity counted in a gamma counter, and residual binding with (%B) and without competitor (%B₀) determined as before. Percentage inhibition (%I) was calculated as follows:

$$\text{Percentage inhibition} = \%I = \left(1 - \frac{B}{B_0} \right) \times 100.$$

Results

CALF VITREOUS HUMOUR RADIOIMMUNOASSAY

The antiserum dilutions showing maximal binding

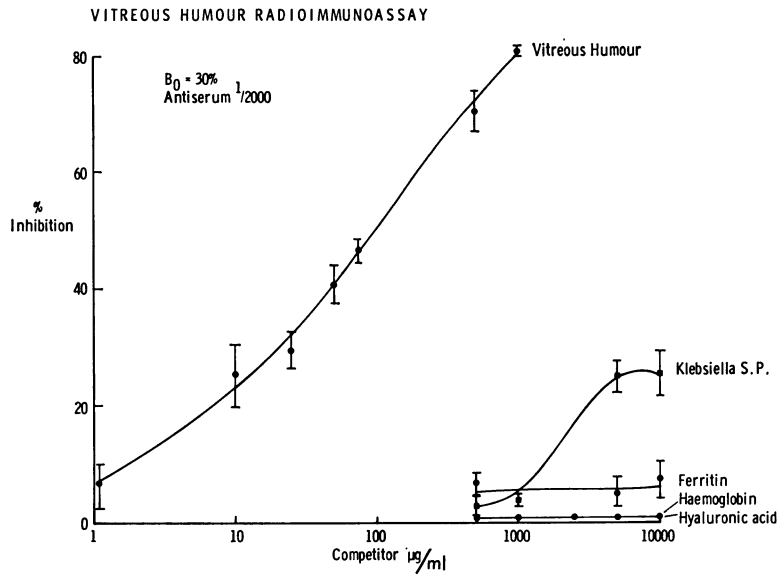


Fig. 1 Inhibition of calf CVH-¹²⁵I tracer binding to rabbit anti-VH-Ig, at a dilution of 1/2000, by increasing amounts ($\mu\text{g/ml}$) of unlabelled vitreous humour, klebsiella sonicate preparation (KSP), ferritin, bovine haemoglobin, and hyaluronic acid. Bars represent means \pm standard errors.

were selected for inhibition studies and were obtained from radiobinding curves prepared for each batch of radiolabelled vitreous humour. RIA studies, with 2 dilutions being used at 1/2000 ($B_0 = 30\%$) and 1/840 ($B_0 = 54\%$) respectively, of A2 preparations of rabbit anti-VH-Ig with calf-¹²⁵I are shown in Figs. 1 and 2.

At 10 000 $\mu\text{g/ml}$ of competitor, klebsiella extract (KSP) showed 26% (Fig. 1) and 30% (Fig. 2) inhibition of vitreous humour binding respectively, while ferritin inhibited less than 10% and bovine haemoglobin, hyaluronic acid, and streptococcus

antigen had no inhibitory effect. The difference in inhibition between KSP and ferritin, when B_0 equals 54% (Fig. 2) is statistically significant ($t = 20.56$, $p < 0.001$). Lower concentrations of competitor produced correspondingly lower levels of inhibition.

COW VITREOUS HUMOUR RADIOIMMUNOASSAY

RIA studies with rabbit anti-VH-Ig preparations, obtained from 2 separate bleeds (A2 and A1), binding to cow vitreous humour are shown in Figs. 3 and 4.

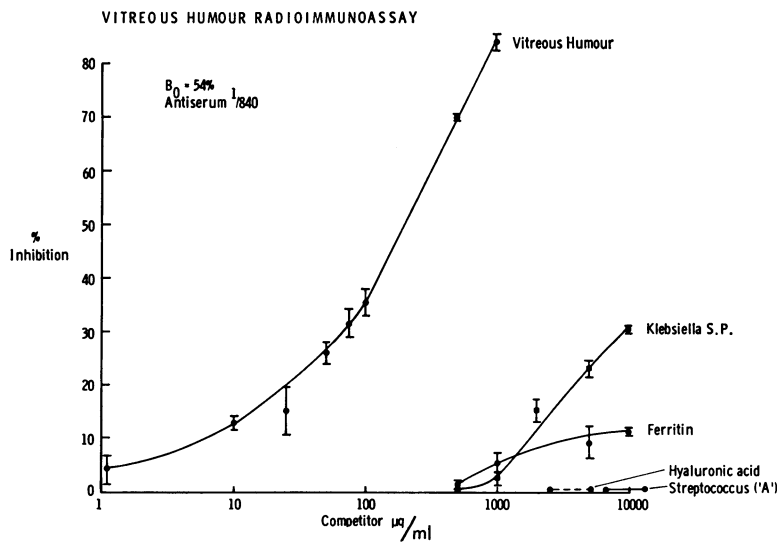
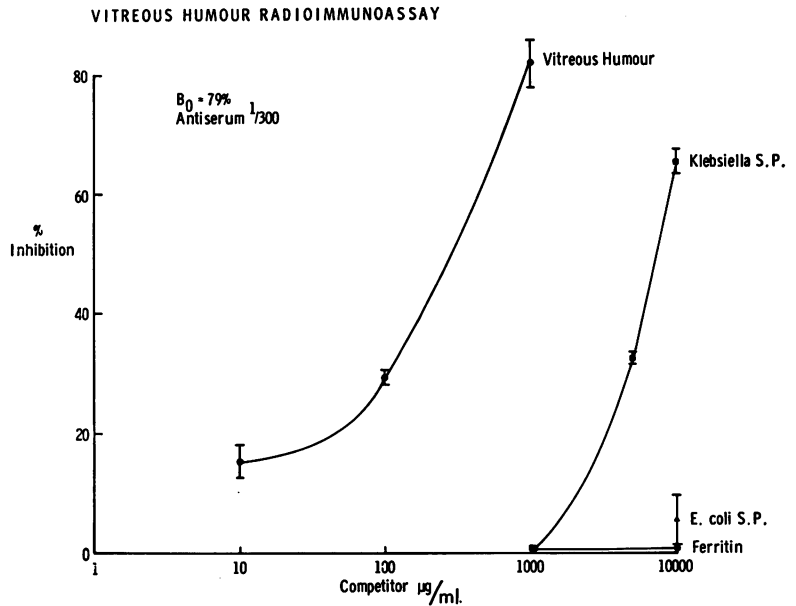


Fig. 2 Inhibition of calf CVH-¹²⁵I tracer binding to rabbit anti-VH-Ig, at a dilution of 1/840, by increasing amounts ($\mu\text{g/ml}$) of unlabelled vitreous humour, klebsiella sonicate preparation, ferritin, hyaluronic acid, and streptococcus A antigen. Bars represent means \pm standard errors.

Fig. 3 Inhibition of cow CVH-¹²⁵I tracer binding to rabbit anti-VH-Ig (A2), at a dilution of 1/300, by increasing amounts ($\mu\text{g/ml}$) of unlabelled vitreous humour, klebsiella and *E. coli* sonicate preparations, and ferritin. Bars represent means \pm standard errors.



At 10 000 $\mu\text{g/ml}$ of competitor, klebsiella extract (KSP) showed 65.5% inhibition of vitreous humour binding by anti-VH-Ig (A2) at a dilution of 1/300 ($B_0=79\%$), while an equal amount of *E. coli* extract produced 4.8% of inhibition, and ferritin had no effect (Fig. 3).

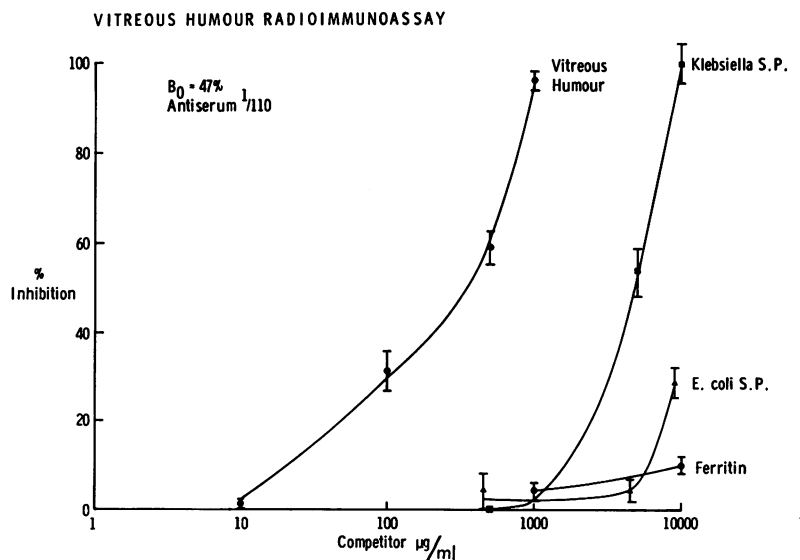
Another preparation of anti-VH-Ig (A1), at a dilution of 1/110 ($B_0=47\%$), showed 100% inhibition with 10 000 $\mu\text{g/ml}$ of klebsiella sonicate preparation (KSP), and this difference is statistically significant when compared with all other competitors

($t=11.76$, $p<0.001$). An equal amount of *E. coli* sonicate preparation showed 28% inhibition and ferritin 10%. Lower concentrations of competitor again produced correspondingly smaller degrees of inhibition.

Discussion

Antigenic similarities between vitreous humour and klebsiella micro-organisms were suggested by the studies described in the previous paper, namely,

Fig. 4 Inhibition of cow CVH-¹²⁵I tracer binding to rabbit anti-VH-Ig (A1), at a dilution of 1/110, by increasing amounts ($\mu\text{g/ml}$) of unlabelled vitreous humour, klebsiella, and *E. coli* sonicate preparations and ferritin. Bars represent means \pm standard errors.



binding studies with labelled vitreous humour. Rabbit antisera raised against klebsiella were found to bind vitreous humour to a greater extent than sera raised against other bacterial or viral antigens. Radioimmunoassay studies described here show that klebsiella antigens directly inhibit the binding of vitreous humour antigen to antivitreous humour antibodies. A similar preparation from another Gram-negative micro-organism, *Escherichia coli*, showed a lower level of inhibition, while other mammalian antigens, such as ferritin, hyaluronic acid, and bovine haemoglobin showed little or no inhibitory effect. A bacterial preparation from a Gram-positive micro-organism, streptococcal group A antigen, also had no inhibitory effect on the binding of vitreous humour antigen by vitreous humour antiserum. The klebsiella preparation has greater inhibitory effect when cow rather than calf vitreous humour is used in the radioimmunoassays. This lower level of inhibition may either represent differences in vitreous humour composition between young and old animals or could be due to the antiserum raised in the rabbit, which had been immunised with vitreous humour obtained from eyeballs of old cows.

Cross-reactivity in radioimmunoassay systems depends on the B_0 value, which arises from the relationship between bound and unbound fractions of radiolabelled tracer and affinity of antibodies in the antiserum used in the assay system. Immunochemical studies using haptenic systems have shown that the higher affinity antibodies interact to a greater extent with antigen analogues than lower affinity antibodies isolated from the same serum.¹² The relationship of the B_0 value to cross-reactivity is therefore the result of a very complex dynamic equilibrium between antibody affinity, amount of tracer, and the affinity of the competitor for the antibody binding site. At present there are no satisfactory physicochemical models for multisite antigen-antibody systems, while precise measurements of affinity constants for these interactions are lacking. Therefore, the assessment of cross-reactivity in such systems requires caution, and comparisons can be made only between the different competitors. The data presented here consist of comparisons between the homologous antigen, vitreous humour, and a number of heterologous antigens, each having a varying degree of biochemical homogeneity and immunological heterogeneity. The preparation from klebsiella micro-organisms was found to compete for antivitreous humour antibodies, compared to other antigens of similar heterogeneity, and one possible explanation is that klebsiella micro-organisms have antigens which resemble some vitreous humour components.

Klebsiella are non-motile, capsulate, Gram-negative bacilli of the family Enterobacteriaceae, frequently found in the lower gastrointestinal tract of man. Investigations into the composition of capsular polysaccharides of klebsiella have demonstrated the presence of glucuronic acid and various monosaccharides such as mannose and galactose,¹³ and a similar polysaccharide composition has been described for vitreous humour.¹⁴ Lipopolysaccharides extracted from Gram-negative bacteria have been shown to specifically inhibit lymphocytotoxic activity of various HLA antisera,¹⁵ and it was suggested that lymphocytotoxins evoked by bacterial antigens could affect the host's own tissues. Thus cross-reactive antibodies, produced after bacterial infection with micro-organisms such as klebsiella, which bind to vitreous humour antigens, as suggested by this study, could be involved in the development of anterior uveitis. The acquisition of an enteric flora has been suggested as a possible cause for the development of isohaemagglutinins in neonatal animals, including man,¹⁶ and thus the appearance of cross-reacting antibodies against other tissues, such as vitreous humour, could also occur after infection of the gastrointestinal tract with Gram-negative micro-organisms.

Antibodies produced after an infection by klebsiella could bind to tissues containing large quantities of cross-reacting material such as vitreous humour and other eye antigens, activate the complement cascade, and produce an inflammatory response. Acute anterior uveitis could be a manifestation of such a process, possibly through the adjuvant effect of some concurrent infection.¹⁷ In experimental animal models, such as adjuvant arthritis, a systemic disease is produced which is characterised by arthritis of peripheral joints and spinal column, together with a nongranulomatous uveitis.¹⁸ It has been suggested that adjuvant-induced uveitis is probably the result of an immunological response of the hypersensitivity type to bacterial antigens,^{19 20} and the demonstration of cross-reactivity between klebsiella micro-organisms and vitreous humour in this study is consistent with such a concept.

It is suggested that uveitis in patients with ankylosing spondylitis could be produced by antibodies evoked after gastrointestinal infection by Gram-negative bacteria.

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