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Cross reactivity between *Klebsiella pneumoniae* and ocular tissue

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Summary
Klebsiella pneumoniae has been implicated as a possible aetiological agent in ankylosing spondylitis and acute anterior uveitis. Cross-reactivity between antigens of klebsiella and bovine vitreous has been reported. In the present study sera from rabbits immunised with *Klebsiella pneumoniae* was tested against fresh guinea pig and human ocular tissues using immunodiffusion and immunofluorescent methods. No cross-reactivity between klebsiella and the ocular tissues used could be demonstrated by these techniques.

Resume
Klebsiella pneumoniae est designe comme un eventuel agent etislogigue de la spondylite ankylosome et de l'uveits aigue. La contre-reaction entre les antigenes de klebsiella et les anticorps de la bovine vintreuse a ete etudice. Dans cette etude, le serum de lapin ammunise a l'aide de *Klebsiella pneumoniae* a ete tests contre les tissue oculaires frais de cobaye et d'homme par les methodes d'immunodiffusion et d'immunofluorescence.

Ces techniques m'ont donne aucune contre-reaction entre klebsiella et les tissus oculaires utilises.

Introduction

The aetiology of acute anterior uveitis is not known in many cases, even when they are thoroughly investigated. Perkins in 1961[1] was able to assign a diagnosis to 18% of his 360 female cases and 55% of his 536 male cases.

The disease is often seen associated with rheumatic diseases such as ankylosing spondylitis, Reiter's syndrome and Still's disease. There is a higher incidence of chronic prostatic infection in males and acute anterior uveitis may occur in ulcerative colitis and Crohn's disease.

Attempts to culture infective agents from aqueous and other parts of the body fluids and tissue have met with no success. The association of acute anterior uveitis and ankylosing spondylitis has been the subject of research in an effort to find the aetiology of either disease. It is said that the incidence of acute anterior uveitis in patients suffering from ankylosing spondylitis is 30%[2]. With the discovery of the HLA system, an association was made between ankylosing spondylitis and HLA-B27. This antigen is present in 90% of ankylosing spondylitis patients as opposed to 8% of the control Caucasian population.[3,4]

This same antigen has been associated with acute anterior uveitis being found in 55% of patients compared to 4% of the controls[5]. There is a suggestion from previous findings that there may be an immune response gene closely linked to the HLA-B locus that determines susceptibility to acute anterior uveitis and ankylosing spondylitis.

Rahi[6] has made an attempt to explain the possible mechanisms by which carrying of HLA-B27 tissue type may increase the susceptibility to acute anterior uveitis. This mechanism includes the theory of molecular mimicry which suggests that the carriage of certain micro-organisms in some systems of the body makes some individuals susceptible to disease if the micro-organisms carry components whose molecular structure mimics one of the HLA antigens. This would lead to an inability of the host to mount a normal immune response to the organism, or the infection by the organism in such an organ may set up reactions in tissues that have similar antigenic properties to the stimulus.

Cross-reactivity and antigenic similarities were found between lymphocytes of HLA-B27 positive patients and klebsiella organisms[7,8,9]. This cross-reactivity has been previously demonstrated between gram negative bacteria and antigens of vertebrates [10,11,12,13]. Some authors however have been unable to demonstrate cross-reactivity between *Klebsiella pneumoniae* and lymphocytes of HLA-B27 positive patients[14].

Consequent to the similarities found between lymphocytes of HLA positive patients and klebsiella organisms, several authors have studied the occurrence of the micro-organism in patients with ankylosing spondylitis and in patients with anky-

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losing spondylitis with uveitis. Ebringer[15,16] found klebsiella aerogenes in the faeces of patients with the active disease more frequently than in those with inactive disease and controls. The authors suggested this micro organism may play a role in the aetiology of ankylosing spondylitis.

When ankylosing spondylitis was associated with acute anterior uveitis 76% of patients had *K. pneumoniae* in their stools as against 30% of ankylosing spondylitis patients without acute anterior uveitis.[17] A similar observation was made by White *et al.*[18] though in their cases, faecal carriage was found only in the first and second week of onset of the disease. Eastmond[19] found that the presence of klebsiella aerogenes in the gut may be related to the development of acute anterior uveitis or peripheral synovitis but found no relationship between this organism and the clinical activity of the spinal disease. In some studies Warren[20,21] and Mawle[22] did not find any association between the faecal carriage of klebsiella and the degree of activity of ankylosing spondylitis nor was there any increase of faecal carriage of klebsiella in uveitis patients.

Also in a large series of patients, Beckingsale[23] found no increase in the carriage of *K. pneumoniae* in acute anterior uveitis patients when compared to controls.

Studies using radioimmunoassay have shown cross-reactivity between *K. pneumoniae* and bovine vitreous humour which suggests that klebsiella micro-organisms may carry antigenic determinants which resemble vitreous humour antigens.[24,25]

If the klebsiella organism has an aetiological role to play in acute anterior uveitis and as suggested, the mechanism may be that of molecular mimicry, it should be possible to establish the antigenic similarity of the organism with anterior uveal tissue by immunofluorescence and immunodiffusion studies.

The purpose of this study was to find out if there are indeed any antigenic similarities between *K. pneumoniae* and tissues of the anterior segment by using anti-klebsiella serum, obtained by immunising rabbits with whole killed bacteria and sonicate preparations of the bacteria, and testing the sera with extracts of ocular tissue for immunodiffusion and against fresh sections of human and guinea pig eyes for immunofluorescent studies.

Materials and methods

Preparation of rabbit antisera against Klebsiella pneumoniae

The Antigen — *Klebsiella pneumoniae* was obtained from a faecal isolate on September 24, 1981 at the University of Iowa Hospitals and Clinics. The strain was *K. pneumoniae* 565. It was cultured on L-agar containing 10 gm typhone and 5 gm yeast extract in 5 mg NaCl at a concentration of 15 gm agar/titer.

Sonicate Preparation — The bacterial culture was made in phosphate buffered saline suspension to make a concentration of 10^{10} cells/mm^3. The required immunising volume was ultrasonicated when needed and used to immunise the rabbit with the regime below.

Whole Klebsiella — Organism suspension was made by harvesting the colonies in 10 ml phosphate buffered saline to make a bacterial concentration of 10^3 cells/mm^3. Fifteen drops of 37% formaldehyde was added and shaken. This preparation was stored at 4°C and used for immunisation as required.

Rabbit immunization

Four New Zealand white adult rabbits in which ocular disease had been excluded by biomicroscopy were used. Their weights varied between 4.0 kg and 4.5 kg. Two of the rabbits received the whole killed organism while the other two were immunised with the sonicate preparation. The immunisation schemes for the four animals were the same and consisted of:

1. Subcutaneous injection of 0.3 ml bacterial suspension twice at 3-day intervals.
2. Three days after the second injection, 0.4 ml bacterial suspension was injected intramuscularly at four sites using buttocks and shoulders, using 0.1 ml at each site. This was repeated four times at 3-day intervals. Thirteen days after, the animals were test bled. Their sera were positive only in 1/10 to 1/20 dilution only. The animals were then given 0.1 ml bacterial suspension intravenously by two injections. Ten days later, test bleeding showed the animals' sera positive at the following dilution by agglutination and precipitation tests:

Animal	Agent for Immunization	Active Serum
A	Whole Klebsiella	1 in 256
B	Whole Klebsiella	1 in 512
C	Sonicated preparation of Klebsiella	1 in 128
D	Sonicated preparation of Klebsiella	1 in 128

Preparation and staining of tissue sections by indirect immunofluorescent method

1. Tissues were obtained from freshly enucleated guinea pig and eyebank human tissues and iridectomy specimens were also examined.
2. The tissues were embedded with OCT compound (a commercial cryostat compound from Ames Co., Division of Miles Laboratories, Inc., Elkhart, Indiana) and snap frozen at low temperature with carbon dioxide. Specimens were stored at -20°C until required.
3. Fresh frozen sections were cut in a low temperature cryostat at 5μ thickness. The cut sections were transferred to a slide with easily labelled frosted ends with a brush. The limits of the specimen were marked with a diamond tip pen. This helped later to form a well to hold the antisera and also simplified locating tissue fragments during examination with the fluorescent microscope.
4. The slides were air dried for 15 minutes and washed in a histological staining dish in a phosphate buffered saline (PBS) for 3 minutes to remove unbound protein. Slides were then immersed in 95% ethyl alcohol for 20 minutes for fixation and then washed in PBS for 5 minutes three times to remove the fixative.
5. Excess PBS was wiped off and slides were transferred to a moist chamber for staining. In the moist chamber, sections were treated with 1/10, 1/20 and 1/40 dilutions of either active or control rabbit sera for 30 minutes. (Active sera = Positive Rabbit anti-Klebsiella serum and Control sera = Pre-immune rabbit serum). Excess serum was washed off with PBS with a squirt bottle. Slides were placed in a Coplin jar for a 15-minute wash in PBS, the control slides were separated from active serum slides.
6. Slides were treated again with fluorescein conjugated goat antirabbit IgG (catalog no. 12-12-0081, lot no. 22055, Cooper Biomedical, Malvern, Pennsylvania, 19355) in 1/10, 1/20, 1/40 and 1/80 dilutions in the moist chamber. Excess conjugated serum was washed off the slides and rinsed for 15 minutes in PBS.
7. The slides were then cover-slipped with immunomount and examined with a Leitz fluorescent microscope.

Eighteen slides were made initially to determine the best serum dilution that would give the most significant difference in fluorescence between control and active sera, that is, active serums 1/10, 1/20 and 1/40 were treated with fluorescein conjugated serum in these dilutions: 1/10, 1/20 and 1/40. Also control sera were treated as above.

Because there was no significant difference in any set of slides, it was arbitrarily decided to use serum dilution 1/20 and 1/40 for the experiment to minimize nonspecific staining.

There was a lot of tissue autofluorescence especially from sclera, cornea and ciliary body. This was minimized in subsequent slide preparation by counterstaining the slides with 0.1% Evan's blue for 15 minutes, excess was washed off and rinsed twice for 10 minutes in PBS, cover-slipped and examined with fluorescent microscope.

Immunodiffusion tests (by Ouchterlony techniques)

Serum - The active sera against the sonicate klebsiella preparation was used and the pre-immune serum of the rabbits was used as the control.

Preparation of tissue extracts

Recently killed albino rabbit eyes were used. After enucleation, dissection was done as described by Perkins and Wood.[26] After careful separation, the tissues (lens, cornea, iris, ciliary body, sclera, vitreous and choroid) were weighed and a 10% wet weight solution made of them by grinding in a tissue grinder, centrifuged and the supernatant used. Then, 5μl of each tissue extract and sera were added to the wells of Ouchterlony plates.

The plates were left in a moist chamber for 24 to 48 hours. The resulting precipitation lines were noted.

Results

Indirect fluorescent test

The rabbit antiserum against whole killed klebsiella obtained from animal A was able to agglutinate klebsiella suspension up to a dilution of 1 in 256. The serum obtained from rabbit B was able to agglutinate klebsiella suspension up to a dilution of 1 in 512, their control sera showed no agglutination at 1 in 4 dilution. When both sera were used in dilution 1 in 10, 1 in 20 and 1 in 40 for the indirect fluorescent test, there was no difference in tissue fluorescence between active and

control sera. Careful examination of the cornea, iris, ciliary body, sclera and vitreous demonstrated no appreciable difference in fluorescence.

The slides of human ocular tissue showed autofluorescence which disappeared when counterstained with 0.1% Evan's blue dye. In each of the slides showing the cornea, ciliary processes, schlera tissue, lens and vitreous, there was no difference in staining between the tissue stained with active and inactive sera.

The klebsiella organism however showed marked fluorescence with the 1/80 dilution of active serum while there was no fluorescence at 1/80 or lower dilution of inactive serum.

Sera from rabbits C and D immunized with sonicated klebsiella (active) was able to agglutinate the klebsiella suspension at a dilution of 1 in 128 while the animals pre-immune sera (inactive) did not agglutinate the suspension.

Therefore, anti-klebsiella sera from these animals did not bind to any tissue in the anterior segment and vitreous of the guinea pig and human specimens.

Immunodiffusion

Anti-Klebsiella serum (sonicate preparation) gave precipitation line to klebsiella sonicate preparation alone. No precipitation lines were formed with extracts of other ocular tissues. The control serum formed no precipitation line with the klebsiella suspension after 48 hours.

Discussion

The experiment did not reveal any binding affinity or site for anti-klebsiella serum in ocular tissue which suggests that there was no demonstrable ocular tissue with similar antigenic property to *Klebsiella pneumoniae*.

The association of HLA-B27 tissue type to acute anterior uveitis[5] has led to several hypotheses, e.g., the immune response genes, the receptor model hypothesis and a third mechanism - the molecular mimicry theory. The evidence that there is increased faecal carriage of klebsiella in HLA-B27 positive patients with ankylosing spondylitis, the cross-reactivity between klebsiella species and lymphocytes of HLA-B27 positive patients and the increased carriage of faecal klebsiella in ankylosing spondylitis patients who develop acute anterior uveitis over controls, have implicated klebsiella as an aetiological agent in acute anterior uveitis. The cross-reactivity between bacteria and human antigens or tissues, e.g., group A streptococcal cells and human heart, [27,28] has been known to be a cause of pathogenicity[11].

If klebsiella causes uveitis by molecular mimicry, it should be possible to demonstrate an affinity of anti-klebsiella serum to ocular tissue. We have been unable to demonstrate this by fluorescent immuno-histophathology. There was no difference in results when antigen (Klebsiella) was ultrasonicated or killed with formaldehyde as demonstrated by Avakian *et al* [24].

The failure to detect cross-reactivity may be due to some factors such as:

1. *Choice of substrate* — The tissues tested in this experiment were derived from guinea pig and human eyes whereas the previous experiments have used bovine and rabbit vitreous. We did not use vitreous as a substrate because the vitreous is rarely involved in acute anterior uveitis and any antigenic similarity between klebsiella and vitreous would seem an unlikely mechanism for the production of an anterior uveitis. A species difference is possible. Welsh *et al.* found that their klebsiella preparation has greater inhibitory effect when cow rather than calf vitreous humour was used in the radioimmunoassays. We feel that the use of human tissue as in this experiment is more relevant to the disease process than bovine vitreous.
2. *Method of detection of cross-reactivity* — Immunodiffusion, although not as sensitive a method as the radio-immunoassay has been used by previous authors[26,29,30] to demonstrate cross-reactivity between ocular and non-ocular tissue, one would expect to be able to demonstrate common antigens between anti-klebsiella serum and ocular tissues by this method if they existed. It might however not be a sufficiently sensitive test. The indirect fluorescent test has been a very reliable technique, which has been used to demonstrate similar antigens in ocular and systemic tissue.[26] If cross-reactivity was present in the samples of serum and tissue used it would have to be very weak not to show by this method. It can be concluded that no cross-reactivity is present between the klebsiella sera we used and ocular tissue.

Although an association between klebsiella and acute anterior uveitis has been found by Ebringer *et al.*, some workers, for example Bec-

kingsale *et al.* and Brewerton have been unable to reproduce the association. In their large group of patients (153 patients and 47 controls) Beckingsale *et al.* found no increase in the carriage rate of klebsiella in acute anterior uveitis patients as compared to controls, even when the patients were grouped into HLA-B27 status, sex or the presence of ankylosing spondylitis. Even if the observed association between gram negative bacteria and ankylosing spondylitis is true, it may not necessarily be the same mechanism operating for acute anterior uveitis.

The increased affinity of anti-klebsiella serum for bovine vitreous does not necessarily explain cross-reactivity phenomenon as a mechanism for acute anterior uveitis since the vitreous hardly ever becomes involved in acute anterior uveitis.

Our study does not support the hypothesis that klebsiella plays a role in the aetiology of acute anterior uveitis.

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