HLA-B27, Klebsiella pneumoniae, and the relation to acute anterior uveitis

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Acute anterior uveitis (AAU) is a disease of unknown etiology, although it is frequently associated with various autoimmune diseases. It has recently been shown in patients with ankylosing spondylitis (AS), a disease often associated with AAU, that antiserum raised against a particular isolate of Klebsiella pneumoniae would cross-react with lymphocytes possessing HLA-B27. The present study was performed to evaluate the response of lymphocytes of patients to K. pneumoniae and to determine the correlation with HLA-B27. The circulating immune complex levels in the serum of the patients were also determined for correlation with the presence of HLA-B27 antigen. We were unable to demonstrate this cross-reactivity in the samples of AAU patients; however, we did find an increased immune complex level that was independent of HLA-B27 antigen. From our results, we conclude that although AAU is clinically associated with AS, the role of K. pneumoniae in these disease states remains unclear.

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Key words: HLA-B27, Klebsiella pneumoniae, acute anterior uveitis (AAU), lymphocyte stimulation, immune complexes, neutrophil function, Immunobeads

The pathogenesis of acute anterior uveitis (AAU) can sometimes be directly attributed to an infecting microorganism; however, in the vast majority of cases, the etiology is unknown. AAU is frequently associated with autoimmune diseases such as ankylosing spondylitis (AS), Reiter's disease, Crohn's disease, and ulcerative colitis. Various immunologic studies have reported that in active cases of AAU, patients have exhibited a T cell lymphopenia and a temporary increase in B lymphocytes.1 In addition, active cases of uveitis often show positive leukocyte-migration inhibition and lymphocyte blast formation, which suggest the presence of cell-mediated immunity in these patients.2 Further studies have indicated the role of cellular immunity in the laboratory models of uveitis by passive transfer of experimental allergic uveitis3 and virus-induced uveitis.3,4 It has recently been shown that rats consistently develop uveitis after a systemic injection of endotoxin. Circulating immune complexes have been implicated as playing a role by effecting a change in ocular vascular permeability, which conceivably may lead to uveitis.5-7

A large amount of data has been accumulated on the association between histocompatibility antigens (HLAs) and disease in humans.8 In particular, the HLA-B27 antigen has been found in association with AS. Recent work has postulated that the microorganism Klebsiella pneumoniae also plays an important role in the pathogenesis of disease.9-13

The present study attempts to demonstrate cellular immune reaction of lymphocytes of
AAU patients stimulated with various sources of *K. pneumoniae*. A possible correlation between the presence of HLA-B27 antigen and AAU in the lymphocyte response to the *Klebsiella* antigen is evaluated, since Seager et al. have recently shown an isolate of *Klebsiella* exhibit cross-reactivity with lymphocytes of patients with AS who are HLA-B27 positive.

In this study the lymphocytes of AAU patients HLA-B27 positive and negative were tested in response to this cross-reactive isolate of *Klebsiella* obtained from Seager's group. Also the level of circulating immune complexes and neutrophil function of AAU patients were tested to evaluate the disease entity.

**Materials and methods**

Peripheral blood samples were obtained from seven patients with AAU who were HLA-B27 positive, seven patients with AAU who were HLA-B27 negative, and seven normal healthy donors who were HLA-B27 negative.

**HLA typing.** HLA-B27 antigen was tested by the lymphocytotoxicity assay at the Tissue Typing Laboratory, University of Illinois Hospital.

**Lymphocyte stimulation test.** Approximately 10 ml of blood were obtained under sterile conditions. A double layer with 6 ml of Ficoll-Hypaque (sp. gr. 1.119), prepared by mixing 10 parts of 50% Hypaque (Winthrop Laboratories, New York, N.Y.) with 20 parts of 9% Ficoll 400 (Pharmacia Fine Chemicals, Piscataway, N.J.) as the lower layer and 3 ml of Ficoll-Paque (sp. gr. 1.007; Pharmacia) as the upper layer, was prepared for the separation of lymphocytes and neutrophils. The solutions were layered in 16 by 100 mm round-bottom sterile plastic tubes (Falcon Laboratory, Div. Becton Dickinson & Co., Oxnard, Calif.) and centrifuged at 400 × g for 30 min at room temperature.

After centrifugation two cell zones were isolated: the lower zone consisting predominantly of neutrophils and the upper zone predominantly lymphocytes. The lymphocytes were washed two times with RPMI-1640 medium and then resuspended in RPMI-1640 with 100 mg/ml penicillin, 100 mg/ml streptomycin, and 10% fetal calf serum (FCS). The cell count was adjusted to 1.5 × 10^6 cells/ml. The microtiter plate assay was then performed with phytohemagglutinin (PHA) and pokeweed mitogen (PWM) in dilutions of 1:1000, which gave the optimum response, and with *K. pneumoniae*. Three strains of *Klebsiella* were obtained randomly from fecal samples at the Bacteriology Section of the University of Illinois Hospital Laboratory. The fourth sample of *K. pneumoniae* (Kp-A) was obtained from Dr. A. F. Geczy, New South Wales Red Cross Blood Transfusion Service, Sydney, Australia.

Bacteria were killed by adding 0.25 ml of 40% formaldehyde to a 20 ml suspension of 10^9 organisms/ml in nutrient broth. The bacteria were then washed two times with sterile 0.9% NaCl and stored at −20° C. The bacterial suspension was adjusted to approximately 10^9 organisms/ml, and 0.1 ml of this suspension was added to 0.1 ml of the lymphocyte suspension in the microtiter plates. The solutions of PHA were added to the lymphocyte suspension in the same manner. All processes were carried out under sterile conditions, and the cultures were tested in triplicate. The degree of lymphocyte transformation was measured as counts per minute (cpm) of tritiated thymidine incorporated into the DNA of the proliferating cells. The maximum response of the patient's lymphocytes to PHA, PWM, and each *Klebsiella* strain was compared to those of the controls. Stimulation index (SI) was calculated by taking the mean cpm of the triplicate of each mitogen stimulation and dividing it by the mean cpm of the unstimulated triplicate.

**Immune complex determination.** The level of soluble immune complexes were measured via the immune complex assay that utilizes the murine leukemic cell line, L1210. The detailed procedure will be published elsewhere. The serum immune complex level is recorded as milligram per deciliter of serum.

**Neutrophil function test.** The neutrophils were washed three times with phosphate-buffered saline (PBS). After the final wash, the cells were resuspended in PBS with 10% FCS (PBS-FCS), and the cell count was adjusted to 3 × 10^6 cells/ml. The assay used for evaluation of neutrophil function is based on a method of O'Donnell et al. It simultaneously assesses both phagocytic function (via Immunobeads) and metabolic integrity (via nitroblue tetrazolium (NBT) dye reduction). NBT (Sigma Chemical Co., St. Louis, Mo.) was reconstituted in PBS by adding 6 mg of NBT per milliliter and vortexed vigorously for 1 min, following by filtering of the insoluble residue. Immunobeads (BioRad Laboratories, Richmond, Calif.), which are polyacrylamide beads coated with rabbit
Table I. Results of lymphocyte stimulation test expressed as the stimulation index of B27-positive and B27-negative AAU patients and normal donors

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Mitogens</th>
<th>K. pneumoniae isolates*</th>
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<tr>
<td></td>
<td>PHA</td>
<td>PWM</td>
</tr>
<tr>
<td>AAU-B27 (+) (n = 7)</td>
<td>104.9 ± 44.5</td>
<td>67.0 ± 48.7</td>
</tr>
<tr>
<td>AAU-B27 (-) (n = 7)</td>
<td>77.8 ± 13.3</td>
<td>60.3 ± 13.9</td>
</tr>
<tr>
<td>Normal donors (n = 7)</td>
<td>105.7 ± 26.4</td>
<td>62.6 ± 21.0</td>
</tr>
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</table>

AAU-B27 (+) = B27-positive AAU patients; AAU-B27 (-) = B27-negative AAU patients.
Results expressed as mean ± S.D.
*KpA is an isolate that was previously shown to exhibit cross-reactivity with HLA-B27 lymphocytes from patients with AS; KpB, KpC, and KpD are random isolates from fecal samples from the University of Illinois Hospital Laboratories.

anti-human IgG heavy chain, were then suspended in the NBT solution at a concentration of 150 to 200 × 10^6 beads/ml.
Two hundred microliters of the neutrophil suspension were added to an equal volume of the Immunobead suspension in 12 by 75 mm siliconized glass tubes. The tubes were centrifuged for 1 min at 160 × g. The supernatant was drawn off to remove excess Immunobeads and replaced with an equal volume of PBS-FCS. The tubes were then incubated for 5 min at 37° C. After incubation the pellet was gently resuspended and the neutrophil function determined. This was done by placing 1 drop of the cell suspension on a microscope slide and covering it with a glass coverslip. Two hundred cells were then observed microscopically at a magnification of 400×, and the neutrophils were categorized as totally functional neutrophils (TFNs), partially functional neutrophils (PFNs), or afunctional neutrophils (AFNs). A TFN is a cell that has the ability to ingest Immunobeads, indicating that the cell is capable of phagocytosis. In addition, the ability of the cell to reduce the NBT dye (seen as a color change from yellow to dark purple) correlates with intracellular killing. A PFN is defined as a cell capable of ingesting Immunobeads, but with no reduction of the NBT occurring. This is indicative of a neutrophil that is capable of phagocytosis but lacks the ability for intracellular killing. The AFN is a cell that does not ingest the beads at all; hence, no assessment of its intracellular killing function is possible.

Results

In the lymphocyte-stimulation test, there was no significant difference in the response of the three groups to the mitogens PHA and PWM. Stimulation with the various K. pneumoniae isolates showed that Kp-A produced the lowest blastogenic response as compared to the other three random isolates in the stimulation of AAU with B27-positive and normal donor lymphocytes. Kp-A also produced a low blastogenic response in the stimulation of AAU with B27-negative lymphocytes. Isolate Kp-D produced the highest blastogenic response of all three groups; however, no significant difference was noted in each group's response to any of the Klebsiella isolates (Table I).

The immune complex assay revealed significantly elevated levels present in the serum of both groups of AAU patients when compared to the normal group. The level for B27-positive AAU patients was slightly higher than that of the B27-negative AAU patients (Table II).

The neutrophil function assay showed that the TFN levels were within normal limits in both groups of AAU patients when compared with those of the control group. B27-positive AAU patients had 70.9% TFNs, B27-negative patients had 69.6%, and the normal group had 71.6% (Table II).

Discussion

The extensive data that link specific HLA antigens with particular human disease have led to several hypotheses. One proposed mechanism is the existence of the immune response (IR) genes. These IR genes reside...
on the sixth chromosome, as do the HLA genes, and control susceptibility to disease by determining the strength of the IR to specific antigens. A defect in these genes can result in a defect in the immune reactivity to a particular antigen and thus a possible susceptibility to disease. Specific HLA genes are inherited along with particular IR genes and act merely as markers for specific disease states rather than playing a major role in the pathogenesis of disease.

The receptor model is another possibility, in which cell surface antigens such as HLA antigens act as receptors for viruses or toxins. Patients who possess certain antigens are therefore susceptible to viral infections or the effects of certain bacterial toxins.

A third mechanism is the proposed model of molecular mimicry, that is, the HLA antigens and certain infectious agents are antigenically similar in structure. This antigenic similarity between host and infectious agent may lead to disease in two ways. First of all, disease may result from the host’s inability to recognize the infecting organism as foreign. In this case, the host mounts no IR. On the other hand, an autoimmune reaction could result from a cross-reactive phenomenon. Microorganisms possessing antigens with antigenic structure similar to that of the HLA antigens are not quickly eliminated by the host. When an IR is mounted against these organisms, it can cross-react with the HLA antigens on normal tissue and set off an inflammatory reaction and thus tissue damage.

Recent studies have postulated that the microorganism *K. pneumoniae* may play a role in the pathogenesis of AS in association with the HLA-B27 antigen, through the molecular mimicry model of cross-reactivity.

*K. pneumoniae* is a ubiquitous organism commonly found in the gastrointestinal tract and is pathogenic only conditionally. This organism was first implicated in the etiology of an autoimmune disease in the case of AS.

The initial association of *K. pneumoniae* to AS was that the patients were found to have an increased incidence of this organism in their stools. It was then shown by Seager’s group that antisera made in rabbits against *K. pneumoniae* lysed lymphocytes of AS patients who were HLA-B27 positive but not of those who were HLA-B27 negative or of healthy donors who possess the HLA-B27 antigen. Lymphocyte stimulation tests revealed that the lymphocytes of B27-positive AS patients were significantly reactive to certain isolates of *Klebsiella* antigens than were the lymphocytes of B27-positive or negative healthy controls. This cross-reactivity occurred in only three of 36 isolates. This is probably related to a more specific biomolecular make-up of *K. pneumoniae* within the species, possibly dependent on serotype.

Subsequent work revealed that human monoclonal HLA-B27 typing sera had increased binding activity for *Klebsiella* extracts by hemagglutination. A most recent study has shown that filtrates of *Klebsiella* K43 contain a factor(s) capable of altering a B27-associated cell surface component of lymphocytes from B27-positive healthy donors.

### Table II. Neutrophil function and immune complex levels in AAU-B27 (+), AAU-B27 (−), and normal donors

<table>
<thead>
<tr>
<th></th>
<th>Immunobead/NBT reduction test</th>
<th>Immune complex level (mg/dl)</th>
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<tr>
<td></td>
<td>TFN (%)</td>
<td>PFN (%)</td>
</tr>
<tr>
<td>AAU-B27 (+) (n = 7)</td>
<td>70.9 ± 6.1</td>
<td>10.0 ± 6.6</td>
</tr>
<tr>
<td>AAU-B27 (−) (n = 7)</td>
<td>69.6 ± 4.2</td>
<td>16.4 ± 6.9</td>
</tr>
<tr>
<td>Normal donors (n = 7)</td>
<td>71.6 ± 5.9</td>
<td>15.9 ± 4.4</td>
</tr>
</tbody>
</table>

AAU-B27 (+) and AAU-B27 (−) as in Table I. Values are means ± S.D.
nors and modifying them to be serologically similar to the cells of B27-positive AS patients. 13

On the basis of these data it was proposed that there may be a cross-reactivity between the HLA-B27 antigen and the K. pneumoniae antigen that may play a role in the disease process of AS.

As mentioned, AAU is frequently associated with other autoimmune diseases; approximately 30% of AAU patients have AS or Reiter's syndrome. The HLA-B27 antigen is present in over 80% of these patients. 21 The occurrence of HLA-B27 in all cases of AAU is approximately 58% 22 as compared to an 8% incidence in the general British population. In addition, approximately 25% of the patients with AS develop AAU. 23

In some patients AAU may be the initial presentation of a disease state with a distinct pathogenesis, such as a penetrating injury giving rise to a purulent inflammation, or be a result of a blood-borne infection. However, in the majority, cases are nonpurulent endogenous inflammations of unknown cause. The strong association of AAU with other autoimmune diseases, along with the link to HLA-B27, strongly suggests the possibility of a common pathogenic mechanism occurring in these diseases.

In evaluating the molecular mimicry hypothesis, we compared the response of AAU patients to the above-mentioned cross-reactive isolate of Klebsiella (Kp-A) and to three other random isolates. According to the hypothesis, one would expect that in the lymphocyte stimulation test, the B27-positive AAU lymphocytes would be stimulated significantly less with the cross-reactive isolate than with any of the random isolates. Also, one would expect the cross-reactive isolate to stimulate the B27-positive AAU lymphocytes less than it stimulated lymphocytes from the other patient groups. This would occur because of the proposed similar antigenic make-up of the Klebsiella isolate and the HLA-B27 antigen of the patients. However, the present study failed to demonstrate any significant difference. The AAU patients, both HLA-B27 positive and negative, responded similarly to all the bacterial isolates. There are several explanations for the dissimilarity of our results to those of previous investigators. One possibility is the existence of a subpopulation of patients within the HLA-B27 group who were not detected by our serologic tests; the disease could be limited to those patients. This might explain why a small percentage of those who possess the HLA-B27 antigen contract the associated diseases. There might also be considerable biomolecular variants within the particular species of bacteria. There is also the possibility that AAU and AS are two distinct disease entities and result from two distinct pathogenic mechanisms. Klebsiella may play a role in the etiology of AS and be totally unrelated to AAU. In addition, one must consider that the etiology of the disease is totally unrelated to Klebsiella. In fact, one group of investigators was unable to demonstrate any cross-reactivity in antisera with double specificity for human lymphocytes and K. pneumoniae. 24 Although we were unable to demonstrate a relationship between AAU and Klebsiella, we cannot state with certainty that no such relationship exists because of the small patient population used in our study.

Immune complexes have been under investigation for their role in uveitis. Tissue damage results from immune complexes due to activation of the complement system, which leads to increased vascular permeability and attraction of polymorphonuclear leukocytes. Immune complexes are ingested by these cells, which stimulate the secretion of proteolytic enzymes that damage a variety of tissue components. The coagulation pathway can be activated in immune complex disease and provide yet another source of vasoactive amines and may also lead to the formation of microthrombi and local ischemia.

A recent study has detected elevated circulating immune complexes in AAU patients and correlated the findings with the presence or absence of the B27 antigen. 25 Significant levels of immune complexes were more frequently detected in B27-negative patients. It
was postulated that the dominant immunopathologic reaction in the B27-positive patients is the cell-mediated tissue injury whereas in the B27-negative group, the immune complex-mediated injury related to the absence of the B27 antigens.

Our assay for circulating immune complexes revealed significant elevation above values for normal healthy donors in both groups of AAU patients.

The antigenic component in the composition of these immune complexes has not yet been determined. Whether or not Klebsiella or toxins from this bacteria play a role in the formation of immune complexes is under investigation. One study demonstrated that rats consistently developed uveitis after a systemic injection of endotoxin, and the possibility exists that a cross-reaction between Klebsiella and the B27 antigen might account for a circulating endotoxemia in B27-related diseases.5

As part of our study we also evaluated neutrophil function, which represents a primary defense against bacterial functions. The results showed that both groups of AAU patients had normal functions of phagocytic and intracellular killing capacities.

In conclusion, we assessed the role of K. pneumoniae in patients with AAU in correlation to the presence of the B27 antigen. We were unable to demonstrate cross-reactivity between the B27-positive AAU patient with a particular isolate of K. pneumoniae as was demonstrated in B27-positive AS patients. Therefore the role of Klebsiella with AAU and the B27 antigen could not be established. Circulating immune complexes were found to be elevated in the AAU patients with no correlation to the presence of the B27 antigen.

REFERENCES