

Chlamydial antibody crossreactivity with peripheral blood mononuclear cells of patients with ankylosing spondylitis: the role of HLA B27

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SUMMARY

We have previously reported the association of *Chlamydia trachomatis* with HLA B27⁺ related diseases. To investigate the possibility that chlamydial antibodies serve to localize the immune response in such diseases, we examined the crossreactivity of chlamydial antibodies (rabbit anti-D and anti-L2 serotypes) with peripheral blood mononuclear cells of patients with ankylosing spondylitis (AS) and anterior uveitis (AU) and with human and bovine ocular tissue and cells in culture. Our results indicate a significantly increased percentage binding of chlamydial antibody (D serotype) to the mononuclear cells of HLA B27⁺ patients with AS when compared with HLA B27⁻ patients with AS ($12.9\% \pm 2.2$ versus $5.4\% \pm 2.2$), B27⁺ controls ($5.5\% \pm 1.5$) and B27⁻ controls ($6.1\% \pm 1.0$). There was no significant difference between controls and HLA B27⁺ patients with AU ($6.6\% \pm 1.9$) and B27⁻ patients with AU ($8.7\% \pm 1.1$). This crossreactivity could not be blocked by monoclonal HLA B27 antibody. Chlamydial antibodies (D and L2) crossreact with human and bovine conjunctiva but not uvea, tissue culture derived iris fibroblasts or smooth muscle cells. Our results provide additional support for the concept of crossreactivity between antibodies to microbial agents and peripheral blood mononuclear cells of patients with HLA B27⁺ AS.

Keywords *Chlamydia trachomatis* ankylosing spondylitis anterior uveitis antibody crossreactivity

INTRODUCTION

The association of the HLA B27 antigen with anterior uveitis (AU) and the group of seronegative arthritic syndromes exemplified by ankylosing spondylitis (AS) is now well established (Brewerton *et al.*, 1973; Ehlers *et al.*, 1971; Mapstone and Woodrow, 1974; Wakefield *et al.*, 1983a). The mechanism by which this genetically determined cell surface antigen predisposes to disease has not been ascertained. Several theories have been proposed (Amos & Ward, 1977), one of the most popular envisages an exogenous or infectious agent that interacts with and/or alters the HLA B27 antigen in such a way as to result in an immune response directed against specific target organs such as synovia or uvea. Several Gram negative bacteria (Saari *et al.*, 1980a,b; Ebringer *et al.*, 1977) and *Chlamydia trachomatis* (Amor *et al.*, 1976; Schachter, 1971) have been implicated in diseases

associated with the HLA B27 antigen, including anterior uveitis, ankylosing spondylitis and Reiter's syndrome.

Evidence supporting a role for *Klebsiella* species and a number of other Gram negative microorganisms in HLA B27 related disease has been reported recently. Ebringer *et al.* (1978) have cultured species of *Klebsiella aerogenes* from the faeces of patients with AU and AS and found an association between the presence of these microorganisms and exacerbations in their uveitis. Recently, Avakian *et al.* (1981) have shown crossreactivity between *Klebsiella pneumoniae* antibodies and a crude preparation of bovine vitreous humor. Additionally, using an antibody to *Klebsiella* K43, Seager *et al.* (1979) have demonstrated cytotoxicity to HLA B27⁺ lymphocytes from patients with AS but not to HLA B27⁺ lymphocytes from controls. When we examined this phenomenon using lymphocytes from patients with AU without evidence of AS (Edmonds *et al.*, 1981) only 20% (two out of 10 patients) were susceptible to the cytotoxic effect of *Klebsiella* antisera. This may indicate that the antigen present on the HLA B27⁺ lymphocytes of patients with AS is not present on most HLA B27⁺ lymphocytes of patients with AU. The role of *Klebsiella* species in AU and the B27-related diseases is controversial (Edmonds *et al.*, 1981). *Klebsiella* infection has not been reported to precede reactive arthritis or uveitis. Furthermore, the role of HLA antigens in relation to the immune response to infections with Gram negative bacteria is unknown (Willshaw, 1981).

Chlamydiae are obligate intracellular microorganisms that have been implicated in a variety of diseases including: non-specific urethritis, cervicitis, salpingitis, pneumonitis, conjunctivitis and trachoma (Mitchison, 1980). Phylogenetically, chlamydiae are closely related to Gram negative organisms and have a number of biochemical and structural similarities (Schachter & Grossman, 1981). Genital strains of these ubiquitous microbes have been implicated in the pathogenesis of Reiter's syndrome (Caldwell, Kromhaut & Schachter, 1981), AS, colitis and AU (Schachter *et al.*, 1966). A number of investigators have demonstrated a relationship between antibodies to chlamydiae and the aforementioned diseases (Amor, 1976). The organisms have been cultured from the urethra and joints of patients with Reiter's syndrome and AS (Schachter *et al.*, 1966). Intraocular inoculation into rabbits of chlamydiae isolated from the synovial membrane of a patient with Reiter's syndrome induced uveitis. The same chlamydial isolate induced chronic arthritis complicated by iritis when injected into the knees of rabbits (Ostler, Schachter & Dawson, 1970).

Previous studies from this department have revealed a significant association between HLA B27⁺ AU and lymphocyte transformation to chlamydial antigen (Wakefield & Penny, 1983). HLA B27⁺ AU is characterized by a T cell lymphopaenia during acute iritis which returns to normal levels with recovery (Wakefield *et al.*, 1983b). The mechanisms underlying this reversible T cell lymphopaenia are unknown but may result from infection or an as yet undetected antibody mediated mechanism. In order to further investigate the relationship between microbial antibodies to chlamydiae and HLA B27⁺ lymphocytes we examined the crossreactivity of antibodies to two serotypes of *Chlamydia trachomatis* (D and L2). The results of the present study provide additional evidence of crossreactivity between antibodies to microbial agents and HLA B27⁺ lymphocytes.

MATERIALS AND METHODS

Patient population. Forty-seven patients, 29 males and 18 females ranging in age from 17 to 73 years, were referred to the Uveitis Research Clinic at Sydney Eye Hospital and the Department of Immunology, St Vincent's Hospital, Sydney. Patients were seen independently by a physician and ophthalmologist. All patients had X-rays of their sacroiliac joints. AS was diagnosed using the New York and Rome criteria (Bennett & Burch, 1967; Kellgren, 1962) for that disease. Twenty-nine healthy laboratory personnel, 17 males and 12 females, served as controls.

HLA typing was performed on all patients and controls by the New South Wales Red Cross Blood Transfusion Service, Sydney, using the two-phase NIH lymphocytotoxicity assay for the HLA B27 antigen. A small group of patients and controls were fully typed for HLA A, B and D locus determinants.

Chlamydial cultures. Chlamydial strains were cultured as previously described (Wang &

Grayston, 1971a,b). Briefly, the D strain of *C. trachomatis*, serotype G-1/OT, was grown in the yolk sacs of embryonated hens' eggs. Suspensions from heavily infected 17-day-old embryos were harvested, grown in nutrient broth and semi-purified by treatment with 2 M KCl. The L2 strain, SA-2f, was grown in L929 cells and partially purified by the same procedure. Suspensions were centrifuged and pellets resuspended in 2 ml of sucrose potassium glutamate saline (SPG) and stored at -60°C .

Chlamydial antibodies. Antibodies to two strains of *C. trachomatis* (D and L2) were raised in four rabbits as previously described and pooled (Tavene, Blyth & Reeve, 1965). Antisera were used at a concentration of 1:640 for the D strain and 1:2560 for the L2 strain, these dilutions had been found in preliminary studies to give adequate cell binding and immunofluorescence. In an earlier series of experiments using a chromium release lymphocytotoxicity assay and the method of Seager *et al.* (1979) neither of these antisera were cytotoxic to lymphocytes of patients with HLA B27⁺ AS or AU.

Chlamydial antibody absorption. Antigen suspensions in SPG had particle counts of 1×10^{10} particles/ml. Suspensions were diluted 1:20 with phosphate buffered saline (PBS). Equal volumes, 50 μl , of antigen and their respective antibody were mixed and incubated at 25°C for 30 min. Elementary bodies were then separated from the antisera by centrifugation at 30,000 *g* for 60 min. The absorbed antisera were tested in parallel with unabsorbed anti-D and anti-L2.

Cell preparation. Human peripheral blood mononuclear cells (PBMC) were isolated from 40 ml of heparinized blood by Ficoll-hypaque density gradient centrifugation. Cells were washed three times with PBS and resuspended to a concentration of 5×10^6 cells/ml in Hank's buffered salt solution (HBSS, Flow Laboratories, Sydney, NSW, Australia). 200 μl of the cell suspensions was placed in each of four 10×75 mm plastic test tubes. 5 μl of T11 (Ortho Diagnostic Systems, Raritan, NJ, USA) was added to the first tube. 5 μl of rabbit anti-D and anti-L-2 were added to tubes 2 and 3 respectively. 5 μl of pooled rabbit sera was placed in the control tube to determine nonspecific binding. 5 μl of PBS was added to the final tube to demonstrate fluorescence due solely to the second antibody. Suspensions were mixed by gentle shaking and incubated on ice for 30 minutes with agitation every 10 minutes. Following this, cells were washed twice in 2 ml of cold HBSS with 1% AB⁺ serum and spun at 250 *g* for 10 min. Supernatants were aspirated to approximately 100 μl and the cells were resuspended by gentle shaking. 100 μl of the fluoresceinated second antibody, diluted 1:20, was added to each tube; goat anti-mouse anti-sera (E Y Laboratories, San Mateo, CA, USA) to the T11 tubes and swine anti-rabbit anti-sera (DAKOPATTS a/s) to the second antibody control, normal rabbit sera, anti-D and anti-L2 tubes. The contents of all tubes were mixed and placed on ice for 30 min, with agitation every 10 min. Cells were then washed twice with cold HBSS, resuspended in 0.5 ml of HBSS and kept on ice until analysis on the Coulter EPICS V flow cytometer. In a separate set of experiments PBMC cells were separated into B cells (surface membrane IgG positive cells), monocytes, as determined by forward-angle light scatter in the flow cytometer, and T cells (E rosette positive cells). The purity of the T cell preparation was checked using monoclonal antibodies (T3 and T11).

Flow cytometry. Cells stained with the various antibodies were enumerated with a Coulter EPICS V (Coulter Electronics, Hialeah, FL, USA) flow cytometer. Cells in a flowing stream were illuminated with 400 mW of 488 nm light from a Spectrophysics Argon Laser (Mountain View, California). Light which was scattered by the cells between 1° and 19° (forward-angle scattered light or FALS) was collected and is a measure of cell size. Fluorescein activated by absorption of the laser light by the fluorescein-labelled antibody probe on the cell was detected at 90° angle by a photomultiplier tube after filtering out the scattered laser light with a 515 nm long-pass interference filter. The log integral fluorescence signal (LIGFL) associated with 10,000 lymphocytes was collected by gating on the FALS signal, analysed and stored on disc for subsequent analysis.

HLA antibody blocking. In order to ascertain the possible site of antibody binding to the lymphocyte cell surface, attempts were made to block the binding of chlamydial antibodies by first incubating the cells with monoclonal HLA B27 antisera (a gift from Prof. I. P. C. McKenzie and Dr J. Trapani). 200 μl of the cell suspensions, containing 5×10^6 cells/ml, were placed in 10×75 mm plastic tubes and preincubated with 5 μl of monoclonal HLA B27 antisera (Trapani *et al.*, 1985). A similar set of tubes was run in parallel as a control for the second antibody. Their contents were mixed and the tubes were placed on ice for 30 min with agitation every 10 min. Cells were washed as

previously described. Capping of antibody was accomplished by incubations of 4 h at 37°C. A control set of tubes containing cells and monoclonal HLA B27 were incubated on ice during this period. 5 µl of anti-D and anti-L2 antisera were added to each set of tubes and the assay continued as above.

Tissue immunofluorescence. The ability of chlamydial antibodies to bind to tissue was examined using 5 µ sections of human and bovine eyes, rat liver and stomach, mouse kidney and stomach as well as tissue cultured human and bovine iris and ciliary body fibroblasts, human smooth muscle cells and skin fibroblasts. Briefly, sections were fixed on to microscopic slides and their surfaces flooded with dilutions of the chlamydial antibodies. Following a 30 min incubation at room temperature, the slides were washed with PBS. The second antibody, swine anti-rabbit FITC conjugated antiserum, was added and the slides were incubated an additional 30 min. Excess antiserum was washed free and the slides were examined under a fluorescent microscope (Reichert-Jung Polyvar).

RESULTS

Patients and controls

The 23 patients presenting with HLA B27⁺ AS ranged in age from 21 to 73 years with a mean age of 38.7 ± 11.7 years. The four patients suffering from AS who were HLA B27⁻ ranged from 17 to 35 years with a mean age of 29 ± 4.7 years. The 20 patients with anterior uveitis, ranging in age from 17

Table 1. Percentage of PBMC from patients and controls which bind antibodies to *C. trachomatis*

Patient group	No. in group	<i>C. trachomatis</i> antibodies	
		Anti-D	Anti-L-2
B27 ⁺ AS ⁺	23	12.9 ± 2.2*	14.4 ± 2.2
B27 ⁻ AS ⁺	4	5.4 ± 2.2	8.9 ± 5.7
B27 ⁺ AU ⁺	10	6.6 ± 1.9	10.0 ± 2.1
B27 ⁻ AU ⁺	10	8.7 ± 1.1	13.6 ± 2.2
B27 ⁺ controls	11	5.5 ± 1.5	8.8 ± 2.7
B27 ⁻ controls	18	6.1 ± 1.0	9.5 ± 1.8

AS = ankylosing spondylitis. AU = anterior uveitis. * Mean ± s.e.

There were significant differences in the percentage of anti-D antibody binding between the B27⁺ AS⁺ group and other groups.

B27⁺ AS⁺ versus B27⁻ AS⁺ $P=0.01$

B27⁺ AS⁺ versus B27⁺ AU⁺ $P=0.025$

B27⁺ AS⁺ versus B27⁻ AU⁺ $P=0.05$

B27⁺ AS⁺ versus B27⁺ controls $P=0.005$

B27⁺ AS⁺ versus B27⁻ controls $P=0.005$

There were no significant differences in anti-D or anti-L2 antibody binding between the other groups.

The specificity of the chlamydial antibodies were determined by preincubating the antisera with their respective antigens before incubation with the PBMC of the B27⁺ AS⁺ group. The mean binding of anti-D was decreased to 1.5 ± 0.6 (88%) while anti-L2 was decreased to 2.5 ± 0.6 (84%).

to 70 years with a mean age of 35.7 ± 12.4 years, included 12 with recurrent disease and two presenting with chronic disease; 10 were HLA B27⁺. The remaining 10 patients with anterior uveitis were HLA B27⁻. The control population comprised 11 HLA B27⁺ and 18 B27⁻ subjects, the mean age of this group being 27.6 ± 9.2 years with a range of 18–54 years.

Chlamydial antibody binding

The results of the chlamydial antibody binding study revealed that more PBMC of HLA B27⁺ patients with AS showed binding of the anti-D serotype antibody when compared with HLA B27⁻ patients with AS, patients with AU, and HLA B27⁺ and HLA B27⁻ controls and the differences were significant (Table 1). Figure 1 shows the FALS versus LIGFL pattern from the flow cytometer demonstrating the differences in anti-D antibody binding to the PBMC of a patient with HLA

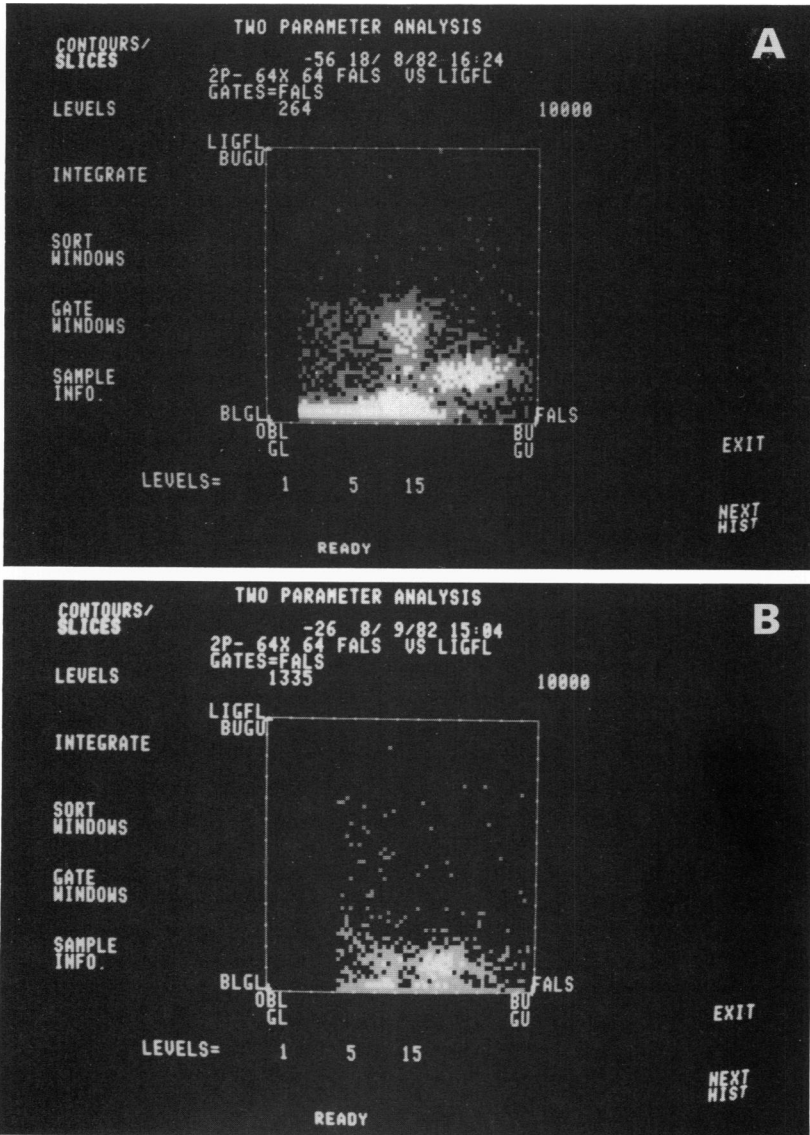


Fig. 1. Two-parameter analysis of chlamydial antibody (anti-D) binding to peripheral blood mononuclear cells. (A) HLA B27⁺ AS⁺ patient showing high percentage of cell binding. (B) HLA B27⁺ control with low-level cell binding.

B27⁺ AS and a HLA B27⁺ control. Antibodies to the L2 serotype bound to slightly more PBMC than did anti-D, but the levels were not statistically different.

The specificity of the chlamydial antibody binding was demonstrated by first reacting the antibody with its respective antigen. This resulted in a decreased overall mononuclear cell binding of 88% for absorbed anti-D and 84% for absorbed anti-L2 (Table 1). The mean percentage binding of normal rabbit sera for the HLA B27⁺ patients with AS was 6.8 ± 1.3 which was not significantly different from the values for HLA B27⁻ patients with AS, AU patients and controls. Chlamydial antibodies were also shown to bind T cells ('E' rosette positive), B lymphocytes (surface Ig positive) and monocytes (identified by FALS) (Table 2). There was no significant difference between antibody binding to T cells and B cells, however, there were significant differences between T cells and monocytes ($P < 0.005$) and B cells and monocytes ($P = 0.001$). Figure 2 describes the quantitative relationship between antibody dilution (serotype D and L2) and the percentage of cells binding antibody from five HLA B27⁺ patients with AS.

The reproducibility of the test was verified by repeatedly examining the antibody-binding characteristics of PBMC from several patients from each disease group and from controls. There was a variation of $\pm 11\%$ between experiments for individuals tested repeatedly on different occasions.

As HLA B27⁺ cells of patients with AS showed increased binding of chlamydial antibodies, we

Table 2. Chlamydial antibody binding to mononuclear cells of patients with HLA B27⁺ ankylosing spondylitis

Chlamydial antibody	Percentage of cells binding antibodies against <i>C. trachomatis</i>		
	T cells (n = 5)	B cells (n = 5)	Monocytes (n = 5)
Serotype D	$22.0 \pm 6.3^*$	13.2 ± 2.3	3.0 ± 0.4
Serotype L2	27.1 ± 7.5	15.9 ± 5.3	2.4 ± 0.8

* Mean \pm s.d.

Chlamydial antibody binding (anti-D and anti-L2) revealed no significant difference between T cells and B cells; however, there were significant differences in antibody binding between T cells and monocytes ($P = 0.005$); and B cells and monocytes ($P = 0.001$).

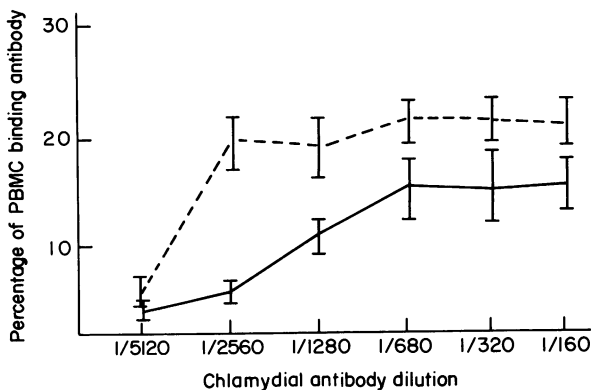


Fig. 2. Relationship between percentage of PBMC binding chlamydial antibody and chlamydial antibody dilution in ankylosing spondylitis patients mean \pm s.e. Titrations of the chlamydial antibodies demonstrated a plateau which eventually declined with increasing dilutions of the antibodies. Titres within the plateau range were used in all experiments. (---) Anti-L2; (—) Anti-D.

Table 3. Effect of preincubation of PBMC with monoclonal HLA B27 antisera on subsequent binding of chlamydial antibody

		Percentage of cells binding antibodies against <i>C. trachomatis</i>			
		HLA B27 ⁺ patients		Controls	
First antibody	Second antibody	AS ⁺ (n=6)	AU ⁺ (n=3)	B27 ⁺ (n=4)	B27 ⁻ (n=4)
—	D	12.8±3.3*	4.7±1.0	3.0±1.1	6.1±5.7
B27	D	12.4±4.1	5.3±0.6	2.4±0.7	5.8±3.0
—	L-2	14.2±4.5	8.6±2.1	8.4±1.0	8.1±1.2
B27	L-2	14.0±3.1	9.7±1.0	7.6±3.0	6.7±2.2

First antibody: preincubation with mouse anti-HLA B27 monoclonal antibody. Second antibody: incubation with chlamydial antibody to serotype D or L2

Preincubation of HLA B27 monoclonal antibody with lymphocytes from patients with AS, AU and controls did not significantly decrease the subsequent binding of chlamydial antibodies anti-D or anti-L2.

investigated whether such antibodies might crossreact with and bind to the HLA B27 antigen. The results of this study of a small group of patients are summarized in Table 3. Prior incubation of the cells with monoclonal HLA B27 antisera, which was demonstrated microscopically, and subsequent capping of the antisera did not affect their subsequent binding of the chlamydial antibodies. In another study, chlamydial antibodies (anti-D and anti-L-2) could be shown by indirect immunofluorescence to demonstrate weak cytoplasmic fluorescence to human and bovine conjunctiva, but not uvea or iris in frozen sections. They did not react with cell lines of human or bovine ciliary body or iris fibroblasts, smooth muscle cells or skin fibroblasts, or with sections of rat liver and stomach or of mouse kidney and stomach.

DISCUSSION

The pathogenesis of HLA B27-related diseases remains elusive despite increasing evidence for the role of a variety of infective agents. The mechanism by which organisms such as *C. trachomatis* may lead to arthritis and iritis is unknown. A genetic predisposition to local mucosal infection (e.g. urethritis) by this obligate intracellular parasite is unlikely (Keats *et al.*, 1980) and most evidence supports an aberrant immune response as causing the disease (Wakefield *et al.*, 1983b).

Our results show significantly increased binding of *C. trachomatis* antibodies of the D serotype but not of the L2 serotype to the mononuclear cells of HLA B27⁺ patients with AS, when compared with HLA B27⁻ patients with AS, patients with AU, and appropriate controls. This may have important implications for the pathogenesis of the disease as D serotypes of chlamydiae cause urethritis and have been implicated in the pathogenesis of Reiter's syndrome, whereas L2 serotypes are responsible for lymphogranuloma venereum and have not been implicated in HLA B27⁺-related diseases.

The antigenic determinant on the mononuclear cell surface responsible for chlamydial antibody crossreactivity has not been characterized. It may not be the HLA B27 antigen itself, as binding this antigen by prior incubation of HLA B27⁺ lymphocytes with monoclonal HLA B27 antiserum did not prevent subsequent chlamydial antibody binding (Table 4). Additionally, if the HLA B27 determinant was the binding site, one would not expect to find a difference in antibody binding

between HLA B27⁺ AS cells and those of HLA B27⁺ controls, unless of course the HLA B27 antigen on the surface of HLA B27⁺ AS cells was in some way different from 'normal' HLA B27⁺ cells. Although physicochemical data does not support the concept of an altered HLA B27 antigen in AS (Karr *et al.*, 1982), Grumet (1983) has recently described possible heterogeneity of HLA B27 antigens, in that an IgM monoclonal antibody (HLA B27 M2) failed to recognize some HLA B27 molecules, and these B27⁻ M2⁻ variants of HLA B27 appear to be associated with AS. Alternatively, chlamydial antibody may bind to the AS gene product proposed by Woodrow (1980). The AS gene may determine genetic susceptibility to AS and be closely linked, although independent of the HLA B27 antigen.

Crossreactivity between antibodies to infective agents and HLA B27⁺ lymphocytes from patients with AS has been intensively studied by several groups, with conflicting results. Geczy, Alexander & Bashir (1980) have found that antisera against certain isolates of *Klebsiella pneumoniae* lyse the lymphocytes of HLA B27⁺ patients with AS and that a culture filtrate of *Klebsiella* K43 can specifically transform the lymphocytes of HLA B27⁺ patients (not previously susceptible to lysis) into cells resembling the HLA B27⁺ lymphocytes of AS patients in their susceptibility to lysis. While this concept of a soluble product from Gram negative bacteria specifically modifying HLA B27 cells is attractive it is yet to be confirmed by other researchers. This process might involve transfer of genetic material, such as occurs in plasmids, that codes for cell membrane antigens in a similar fashion to that hypothesized for chlamydiae. In earlier experiments we failed to detect chlamydial antibody lymphocytotoxicity for HLA B27⁺ PBMC from patients with AS, using a chromium release assay (data not shown). The results of the present study may serve to explain why several groups have failed to confirm the crossreactivity between antibodies to infective agents and HLA B27⁺ cells. Such antibodies against some strains of Gram negative bacteria, as with *C. trachomatis*, may not be cytotoxic, but may be shown to selectively crossreact with HLA B27⁺ cells from patients with AS by other techniques, such as indirect immunofluorescence.

Both chlamydial D and L2 antibodies bound to human and bovine conjunctiva and corneal epithelium in sections as revealed by positive fluorescence but did not bind to any of the cell lines tested. This finding may indicate a mechanism for the localization and pathogenesis of the conjunctivitis (trachoma) and perhaps AU caused by some strains of *C. trachomatis*. AU has been demonstrated to complicate chlamydial conjunctivitis and in some cases developed in the course of conjunctival reinfection or several months after the infection had subsided (Verin, 1980). Chlamydiae have been isolated from the conjunctiva of a patient with Reiter's syndrome and the patient had significantly elevated chlamydial antibodies in his serum (Mattila, Granfors & Toivanen, 1982).

Our failure to demonstrate crossreactivity with uveal cells may be because these cells did not have chlamydial antigen expressed on their surface and/or were not HLA B27⁺. The relationship between the antigen to which the chlamydial antibodies reacts and the HLA B27 antigen remains to be determined. They may not necessarily be spatially related on the cell surface since the HLA B27 antigen might function in selectively allowing expression of a 'chlamydial antigen' or AS gene product on the surface of the cells.

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