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Mimicry of Human Histocompatibility HLA-B27 Antigens by *Klebsiella pneumoniae*

MINORU OGASAWARA, DWIGHT H. KONO, AND DAVID T. Y. YU*

Department of Medicine, University of California at Los Angeles Center for Health Sciences, Los Angeles, California 90024

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Anti-HLA-B27 monoclonal antibody M2, which was relatively specific for human histocompatibility antigen HLA-B27, was used to test several bacteria, some of which could potentially induce chronic arthritis in HLA-B27-positive individuals. Using the Western blot procedure, we observed positive reactions with 80,000- and 60,000-dalton antigens with one strain of *Klebsiella pneumoniae*. Reactivity was not observed with five other monoclonal antibodies which were not reactive with HLA-B27 antigens, nor was reactivity observed with seven other gram-negative bacteria, irrespective of their arthritis-causing potential. To test the validity of our observation, the 80,000-dalton *Klebsiella* cross-reactive antigen was isolated and used to generate an immune guinea pig serum. We found that the reactivity of this guinea pig serum with *Klebsiella* envelopes in an enzyme-linked immunosorbent assay was adversely affected by absorption with HLA-B27-positive cells. Our results support the existence of mimicry between HLA-B27 antigens and bacteria.

The pathogenesis of several arthritis conditions, which are grouped together as the spondyloarthropathies, remains unclear. In the case of Reiter's syndrome, the development of arthritis is very often preceded by a symptomatic episode of infection (3). The responsible infectious organisms which have been identified include *Shigella flexneri*, *Salmonella typhimurium*, *Yersinia enterocolitica*, *Campylobacter jejuni*, and *Chlamydia trachomatis*. It is very unlikely that the arthritis is caused simply by uncontrolled multiplication of these organisms in the host joint areas. Otherwise, the arthritis symptoms would be expected to appear during the episode of disseminated infection and not after the infection has subsided completely. Moreover, none of these organisms has ever been cultured from arthritis joints (8).

In contrast to Reiter's syndrome, the development of another member of the spondyloarthropathy group, ankylosing spondylitis, is insidious and is not preceded by obvious infectious episodes (3). Nevertheless, the bacterium *Klebsiella pneumoniae* has been strongly implicated, as some investigators have observed a higher incidence of *Klebsiella* in the bowel flora of patients whose disease is in an active state (5).

An important investigative clue to be considered for both conditions is that 70 to 90% of the affected patients share HLA type HLA-B27, although this phenotype is present in 10% or less of normal Caucasians (8). Two major hypotheses have been postulated, especially regarding the relationship between *K. pneumoniae* and ankylosing spondylitis. One hypothesis is based on an as-yet-unconfirmed finding that rabbit antisera generated against one strain of *K. pneumoniae* are reactive with lymphocytes of HLA-B27-positive ankylosing spondylitis patients. These sera also react with lymphocytes from HLA-B27-positive normal individuals, if such lymphocytes are first incubated in vitro with supernatants from *Klebsiella* cultures. Lymphocytes from HLA-B27-negative individuals are not reactive, even after incubation with the supernatants. From these data, Seager et al. concluded that the cells of ankylosing spondylitis patients are modified in situ by the bacteria (15). One strain of *K. pneumoniae*, strain K43, has been used repeatedly for generation of antisera, while another *K. pneumoniae* strain, strain K77, is negative. These two strains were included in this study.

In the alternate hypothesis, it is postulated that there is mimicry between the HLA-B27 antigens and the *Klebsiella* cells themselves, with no need for a bacterium-secreted lymphocyte-modifying factor. In previous studies to test for this mimicry, workers used anti-HLA-B27-specific alloantisera and made no attempt to identify the cross-reactive bacterial antigens (1). The disadvantage of using alloantisera is that there is considerable contamination by naturally occurring antibacterial antibodies. With the advent of the monoclonal antibody techniques, the validity of mimicry needs to be examined again. Members of our group have recently generated a panel of monoclonal antibodies against *Yersinia enterocolitica*. We have noticed that one of these anti-*Yersinia* antibodies also cross-reacts with HLA-B27-positive lymphoblastoid cell line cells (9a). However, the avidity of this monoclonal antibody is too low to allow precise biochemical analysis of the cross-reactive bacterial antigens. Other monoclonal antibodies which can conceivably be used for biochemical analysis of the reactive antigens are the monoclonal antibodies which are generated against the HLA-B27 antigens themselves. Indeed, van Bohemen et al. have described the results of experiments in which they used two such monoclonal antibodies, anti-HLA-B27 M1 and anti-HLA-B27 (antibodies M1 and M2, respectively) M2 (18). These authors found that antibody M1 is reactive with a 16,000-dalton (16K) antigen which is present in *Y. enterocolitica* serotype O:9 and in *K. pneumoniae* K21 and K43. Antibody M2 is reactive with a 20K antigen which is present in *Shigella flexneri*. However, van Bohemen et al. used a method which is seldom used outside their laboratory. Furthermore, whole ascites fluids were tested, and no absorption experiments were carried out to ensure that the reactions which were observed were not due to natural antibacterial antibodies which could coexist with the anti-HLA-B27 monoclonal antibodies in the ascites fluids.

In this study we examined these types of bacteria by using monoclonal antibody M2. An enzyme-linked immunosor-

* Corresponding author.

TABLE 1. List of bacterial strains

Strain	Arthritis-causing potential	Reference
K. pneumoniae K43	+	15
K. pneumoniae K77	−	15
Shigella flexneri 7060	+	16
Shigella sonnei 7062	−	7

bent assay (ELISA) and Western blotting were used, and absorption experiments were performed to ensure the validity of the observations. Our results indicated that two antigens in *K. pneumoniae* strain K43 were cross-reactive with HLA-B27 antigens.

MATERIALS AND METHODS

Bacterial strains. *K. pneumoniae* K43 and K77 were kind gifts from Andrew Geczy, Blood Transfusion Center, Sydney, Australia. *Shigella flexneri* 7060 and *Shigella sonnei* 7062 were obtained from the Centers for Disease Control, Atlanta Ga. *Shigella flexneri* 7060 was the cause of an epidemic which was followed by development of Reiter's syndrome in some of the patients (16). On the other hand, *Shigella sonnei* 7062 was the cause of an epidemic which did not induce arthritis (7). The *E. coli*, *Salmonella typhimurium*, and *Y. enterocolitica* serotype O:9 strains used were from our collection. Some of the bacteria used are listed in Table 1.

Monoclonal antibodies. Antibody M2 (6) was purchased from Cappel Worthington Biochemicals, Malvern, Pa. Anti-HLA class I antigen antibody w6/32 (14) was a kind gift from Peter Parham, Stanford University, Palo Alto, Calif. Monoclonal antibodies specific for the lipopolysaccharides of *Yersinia enterocolitica* serotype O:3 were generated in our laboratory by using procedures described elsewhere (12). The other monoclonal antibodies, which were reactive with various lymphocyte antigens, were generated in the laboratory of Paul Terasaki, Department of Surgery, University of California at Los Angeles Center for Health Sciences, Los Angeles. All of the monoclonal antibodies were contained in ascites fluids. Table 2 lists these monoclonal antibodies and their reactive antigens.

Preparation of bacterial antigens. The following three types of bacterial antigens were prepared: Formalin-killed whole bacterial cells, sonicated bacterial envelopes, and Triton X-100-solubilized bacterial envelopes.

Bacteria stored in the lyophilized state were streaked onto Trypticase soy agar. Several colonies of each strain were selected, inoculated into 200 ml of Trypticase soy broth, and cultured at 37°C for 12 h. Formalin-killed whole bacterial cells were prepared as described previously (2).

To prepare sonicated bacterial cell envelopes, cultured bacteria were pelleted by centrifugation at 3,000 × *g* for 10 min at 4°C and then suspended in 10 ml of 10 mM sodium phosphate-buffered saline (pH 7.4) containing 10 μg of DNase per ml and 10 μg of RNase per ml. Samples (1 ml) of the resulting suspension were subjected to sonication at full power for 3 min at 0 to 5°C with a Sonifier cell disruptor (model W1400; Ultrasonics Inc., Plainview, N.Y.). Nondisrupted organisms were removed by centrifugation at 3,000 × *g* for 10 min at 4°C. The disrupted bacterial envelopes were sedimented by centrifugation at 100,000 × *g* for 45 min.

To prepare detergent-solubilized bacterial envelopes, sonicated bacterial envelopes were sedimented by centrifugation and suspended in 200 μl of a solution which contained 1% Triton X-100, 10 mM EDTA, 150 mM NaCl, and 10 mM Tris hydrochloride (pH 7.8). After incubation at 4°C for 15 min, the insoluble material was removed by ultracentrifugation. Reagents were purchased from Sigma Chemical Co., St. Louis, Mo. Protein concentrations were measured by using the method of Lowry et al. (11). The three types of bacterial antigen preparations were stored frozen at −70°C before they were used.

Preparation of lymphocytic antigens for the ELISA. Lymphoblastoid cell lines were cultured in RPMI 1640 medium containing 10% heat-inactivated fetal calf serum (M.A. BioProducts, Walkersville, Md.). To prepare antigens for the ELISA, portions (10^9 cells) of HLA-B27-positive cell line HOM-2 were washed with phosphate-buffered saline and each portion was suspended in 1 ml of a solution containing 0.5% Nonidet P-40 (NP-40) (Calbiochem-Behring, La Jolla, Calif.), 0.2 mM phenylmethylsulfonyl fluoride, and 10 mM Tris hydrochloride (pH 7.4). After 30 min of incubation at 4°C, the insoluble residues were removed by centrifugation at 105,000 × *g* for 60 min. The NP-40-solubilized antigens were precipitated by incubation at −20°C with 9 volumes of 95% ethanol, suspended in sodium carbonate buffer (pH 9.6), and kept frozen at −70°C before they were used.

ELISA of antibody reactivity. The methods used for the ELISA have been described previously (12, 13). The following antigens were used to coat the microtiter plates: lymphocytic antigens prepared as described above (100 μl of preparation per well), Triton X-100-solubilized bacterial envelopes (0.5 μg per well), and Formalin-killed whole-bacterial cells (50 μl of a 1% suspension per well). Antibody reactivity was assessed by using peroxidase-conjugated anti-mouse immunoglobulin M (IgM) (TAGO, Burlingame, Calif.) and peroxidase-conjugated anti-guinea pig IgG antibodies (Zymed, San Francisco, Calif.). Enzyme substrates for peroxidase were *O*-phenylenediamine and hydrogen peroxide. Absorbance was measured with a microelisa autoreader (model MR580; Dynatech Laboratories, Inc., Alexandria, Va.) at a wavelength of 490 nm. The maximum threshold of the autoreader was adjusted to an optical density of 1.5. Samples were assayed in duplicate, and the results were expressed as averages.

SDS-PAGE. For sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis PAGE a 40-μg sample of bacterial envelope protein was incubated at 100°C for 5 min with an equal volume of 2% SDS sample buffer (9). The sample was then electrophoresed in a gel (thickness, 1.5 mm) as described by Laemmli (10), first through a 3.5% acrylamide

TABLE 2. List of monoclonal antibodies

Monoclonal antibody	Specificity	Source or reference
M2	HLA-B27	6
w6/32	HLA class I antigens	14
66-2C9C6	β_2-Microglobulin	P. Terasaki
CBL4	Transferrin receptor	P. Terasaki
CT2	Sheep erythrocyte receptors	P. Terasaki
6B6	Lipopolysaccharide of *Y. enterocolitica* O:3	D. Yu
9B3	Lipopolysaccharide of *Y. enterocolitica* O:3	D. Yu

stacking gel and then through a 10 or 11.5% acrylamide resolving gel. The standard molecular weight markers used were phosphorylase (97.4K), bovine serum albumin (66K), egg albumin (45K), carbonic anhydrase (29K), and β-lactoglobulin (18.4K). The proteins in the gels were visualized by staining with Coomassie brilliant blue R-250.

Western blot analysis. Bacterial envelope components separated by SDS-PAGE as described above were transferred by electrophoresis onto 0.45-μm nitrocellulose membranes (17). The membranes were cut into 3-mm strips, and the success of transfer was demonstrated by staining the proteins on one of the nitrocellulose strips with 1.0% amido black. To block the free binding sites, the other strips were incubated at 37°C for 30 min in a solution containing 3% bovine serum albumin and 50 mM Tris hydrochloride (pH 7.4). Each strip was then incubated in 4 ml of an antibody preparation which was diluted in phosphate-buffered saline containing Tween. Monoclonal antibodies were used at a dilution of ascites fluids of 1:500; guinea pig antibodies were used at a dilution of sera of 1:2,000. Incubation with monoclonal antibodies was carried out at room temperature for 1 h and then at 4°C for 5 h. Incubation with guinea pig antibodies was carried out at room temperature for 1 h. After incubation the nitrocellulose strips were washed extensively. In the case of the monoclonal antibodies, two different washing buffers were compared. The first buffer was a solution containing 0.5% Tween 20 and 50 mM Tris hydrochloride (pH 7.4), and the second buffer was a solution containing 1% NP-40, 150 mM NaCl, and 50 mM Tris hydrochloride (pH 7.4). Only the first buffer was used for the guinea pig antibodies. After washing, the nitrocellulose strips were incubated with 1:1,000 dilutions of peroxidase-conjugated goat antibodies specific for either mouse IgG or mouse IgM (TAGO) or with peroxidase-conjugated rabbit anti-guinea pig IgG antibodies (Zymed). Antibody reactivity was visualized by incubation with the enzyme substrates diaminobenzidene and hydrogen peroxide as described previously (12, 13).

Absorption of antibodies. To absorb antibodies with lymphoblastoid cell line cells, 1 ml of a 1:100 dilution of guinea pig serum or monoclonal antibody in ascites fluid was incubated with 10^8 lymphoblastoid cell line cells at room temperature for 40 min. Then the suspensions was centrifuged at 300 × *g* for 10 min to remove the cells. The lymphoblastoid cell lines tested were HLA-B27-positive cell line HOM-2 and HLA-B27-negative cell lines 8402 and Daudi.

To absorb antibodies with whole bacterial cell antigen or sonicated bacterial envelopes, 200 μl of the former or 1 mg of the latter was used.

Blocking antibody activity in ELISA or Western blot procedures by adding bacterial antigens. Triton X-100-solubilized envelope antigens prepared as described above were used to block antibody reactivity in the ELISA or in the Western blot analysis. For the ELISA 100-μl portions of the antibodies at 1:100 dilutions were incubated with 2 to 16 μg of Triton X-100-solubilized bacterial envelopes, first at 37°C for 60 min and then at 4°C for 3 to 4 h. Any crude sediment was removed by centrifugation at 4,000 × *g* for 10 min, and the supernatants were used for the ELISA.

To block Western blot reactivity, 400 to 500 μg of Triton X-100-solubilized bacterial envelopes was added to 4 ml of a 1:500 dilution of monoclonal antibody. This preparation was incubated at 37°C for 30 min and then at 4°C for 1 h before it was applied to nitrocellulose membrane strips.

Testing anti-HLA-B27 activity by the microcytoxicity method. Standard procedures for complement-dependent dye exclusion microcytoxicity were used, as described elsewhere (Kono et al., in press).

Isolation of *Klebsiella* antigens by preparative SDS-PAGE. A 1-mg sample of sonicated *Klebsiella* envelopes was incubated at 100°C for 5 min with 500 μl of 2% SDS sample buffer, and the sample was then electrophoresed in a gel (1.5 mm thick by 14 cm wide). Proteins were transiently visualized as white bands by incubating the gel with a 0.25 M KCl solution as described previously (13). The sections of the gel at molecular weight ranges of 75,000 to 85,000, 55,000 to 65,000, and 35,000 to 40,000 were excised. The proteins in the gels were eluted as described previously (13).

Immunization of guinea pigs. The eluted antigens were mixed with Freund complete adjuvant. One guinea pig was immunized with the antigens eluted from each of the three sections of the gel. The antigens were given in two portions at 2-week intervals. The animals were bled 1 week after the last injection.

RESULTS

Preliminary analysis for cross-reactivity by using bacterial antigens which were not solubilized with detergents. Formalin-killed whole bacterial cells (Table 1) were tested by using the ELISA with antibody M2 at serial dilutions of 1/50 to 1/400. No reactivity was observed.

Experiments were also carried out to test the effect of absorption with Formalin-killed whole bacterial cells or sonicated bacterial envelopes on the reactivity of antibody M2. In this case, the reactivity of antibody M2 was assayed by the microcytotoxicity method, and the target cells were HLA-B27-positive cell line HOM-2 cells. Absorption with these two bacterial preparations did not affect the reactivity of antibody M2.

Because of these negative results, we reasoned that, if indeed cross-reactivity exists, the determinants are not on the surfaces of the bacterial envelopes and would be exposed by dissociating the envelopes with detergents.

Testing for cross-reactivity with the ELISA by using detergent-solubilized envelopes as antigens. Our experimental approach was to establish an ELISA to quantitate the reactivity of antibody M2 with HLA-B27 antigens. Microtiter wells were coated with antigens prepared from HLA-B27-positive cell line HOM-2 cells. Several dilutions of ascites fluid containing monoclonal antibody M2 were added. Considerable reactivity was observed at dilutions of 1/50 to 1/200. A control IgM monoclonal antibody, antibody 9B3, which was specific only for the lipopolysaccharide of *Y. enterocolitica* serotype O:3, was poorly reactive with the cell line HOM-2 antigens at tne dilutions tested (Fig. 1).

In the next ELISA experiment, antibody M2 was used at a dilution of 1:100. The antigen used to coat the ELISA plates was again a cell line HOM-2 cell extract. Prior to testing, samples of antibody M2 were preincubated with 2 to 16 μg of Triton X-100-solubilized bacterial envelopes. A decrease in the reactivity of the monoclonal antibody was observed when preincubation was with envelopes prepared from *K. pneumoniae* K43 (Fig. 2). Although incubation with envelopes from *E. coli*, *Y. enterocolitica*, and *Shigella flexneri* also caused decreases, such decreases were similar to the decrease obtained if equivalent amounts of Triton X-100 alone were added to the antibody. (The results for the Triton X-100 control are shown in Fig. 2.)

Identification of the cross-reactive antigens in *K. pneumoniae* K43 by using the Western blot technique. Sonicated cell

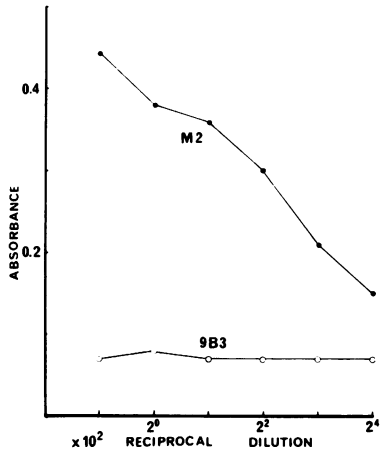

FIG. 1. ELISA of antibody M2, performed by using extracts of HLA-B27-positive cell line HOM-2 cells as antigens. Microtiter wells are coated with cell line HOM-2 cell antigens and then reacted with various dilutions of either monoclonal antibody M2 or monoclonal antibody 93B. Results are determined by the ELISA. Antibody M2 was an anti-HLA-B27 antibody, and antibody 9B3 was an anti-*Yersinia* lipopolysaccharide antibody.

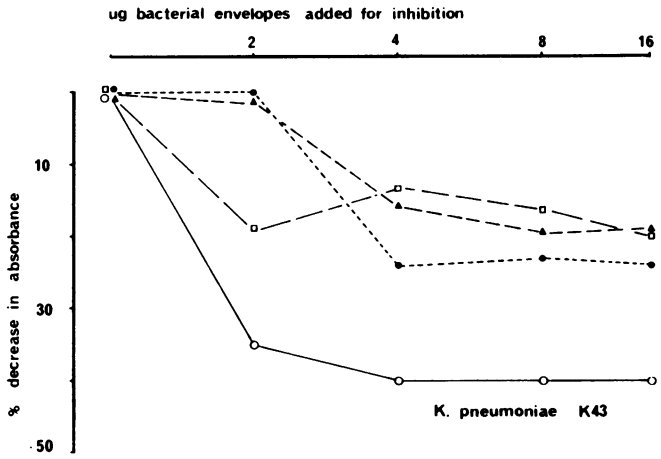

FIG. 2. Inhibition of the reactivity of antibody M2 by using Triton X-100-solubilized cell envelopes from several bacteria. Samples of antibody M2 were preincubated with cell envelopes of several bacteria. These absorbed antibody preparations were tested in the ELISA by using extracts from HLA-B27-positive cell line HOM-2 cells as antigens. Results are expressed as percent decreases in absorbance compared with untreated monoclonal antibody. Symbols: ○, *K. pneumoniae*; □, *Shigella flexneri*; ▲, *E. coli*; ● *Y. enterocolitica*. Inhibition was about 20% when equivalent amounts of Triton X-100 alone were added (data not shown).

envelopes of *K. pneumoniae* K43 were subjected to SDS-PAGE. Coomassie brilliant blue staining showed that there were at least 10 distinct bands (Fig. 3, lane D). Amido black staining of the proteins transferred from the polyacrylamide gels onto the nitrocellulose membranes also showed the same bands. The most prominent bands were at apparent molecular weights of 80,000, 39,000, and 37,000 (Fig. 4, lane A). The reactivity of these electrophoretically separated proteins with antibody M2 was studied and the results depended on the washing buffer used. When 0.5% Tween 20 was used in the washing buffer, two bands (80K and 60K) showed positive reactions. However, when 1% NP-40 and 0.15 M NaCl were used in the washing buffer, only the 60K band was reactive (Fig. 4, lanes B and C).

The blotted nitrocellulose strips were also tested with five other IgM monoclonal antibody (antibodies CBL4, CT2,

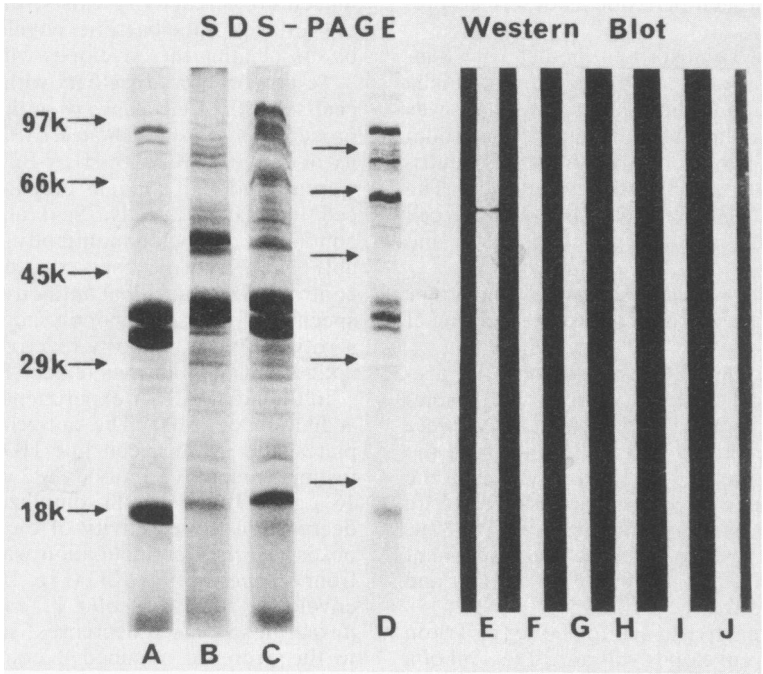

FIG. 3. Coomassie brilliant blue staining of bacterial envelope proteins separated by SDS-PAGE and the results of a Western blot analysis in which these components were used. Lanes A through D show the results of a SDS-PAGE analysis. Lanes E through J show the results of a Western blot analysis. Lanes A and I, *E. coli*; lanes B and F, *Shigella flexneri*; lanes C and H, *Salmonella typhimurium*; lanes D and E, *K. pneumoniae*; lanes G and J, *Y. enterocolitica* and *Shigella sonnei* respectively.

66-2C9C6, 6B6, and 9B3) (Fig. 4, lanes D through H) and one IgG monoclonal antibody (antibody w6/32). The Western blot results showed that these antibodies reacted only very weakly with the *Klebsiella* antigens. These weak reactions were with the 39K and 37K major proteins. Similar weak reactions were also observed with sera from unimmunized mice. The negative reactions with the control antibodies were observed irrespective of whether Tween 20 or NP-40 was used as the detergent in the washing buffer.

Western blot analysis in which other bacteria were used as antigens. As controls for *K. pneumoniae* K43, the following bacteria were also tested by using Western blot analysis with antibody M2: *K. pneumoniae* K77, *Shigella flexneri* 7060, *Y. enterocolitica*, *Salmonella typhimurium*, *E. coli*, and *Shigella sonnei* 7062. Both 0.5% Tween 20 and 1% NP-40 washing buffers were used. More than 10 bands were observed with each bacterial strain when the nitrocellulose membrane strips were stained with amido black. Representative Coomassie brilliant blue-stained polyacrylamide gels are shown in Fig. 3, lanes A through D. The only bacterium which showed significant reactivity with antibody M2 was *K. pneumoniae* K43 (Fig. 3, lanes E through J).

In a separate experiment, the non-HLA-B27-specific monoclonal antibodies listed in Table 2 were tested with the bacteria listed above by using the Western blot technique. No significant reactivity was observed (data not shown).

Testing the validity of the Western blot results by adding inhibitory bacterial antigens or by absorbing the antibodies. The effect of absorption on the reactivity of antibody M2 with the 80K and 60K *Klebsiella* antigens was tested. A decrease in reactivity was observed when absorption was carried out with HLA-B27-positive cell line HOM-2 cells, but not when absorption was carried out with HLA-B27-negative line cell 8402 cells (Fig. 5, lanes A and B). In another experiment (data not shown) no decrease in reactivity was observed when absorption was carried out with a second HLA-B27-negative cell line, cell line Daudi.

In a separate Western blot procedure, 1% NP-40 buffer was used as the washing buffer. As shown above, the predominant reactivity with antibody M2 was observed with the 60K *Klebsiella* antigen. Addition of Triton X-100-solubilized envelopes from *K. pneumoniae* K43 completely inhibited this reactivity (Fig. 5, lane C). Much less inhibition was observed when envelopes prepared from the following strains of bacteria were added instead: *Shigella flexneri* 7060, *Y. enterocolitica*, *Salmonella typhimurium*, *E. coli*, and *Shigella sonnei* 7062 (Fig. 5, lanes D through H).

Isolation of antigens from *K. pneumoniae* K43 by preparative SDS-PAGE. *Klebsiella* antigens were eluted from three sections of a polyacrylamide gel. These sections consisted of the areas at the following molecular weight ranges: 75,000 to 85,000, 55,000 to 65,000, and 35,000 to 40,000. The concen-

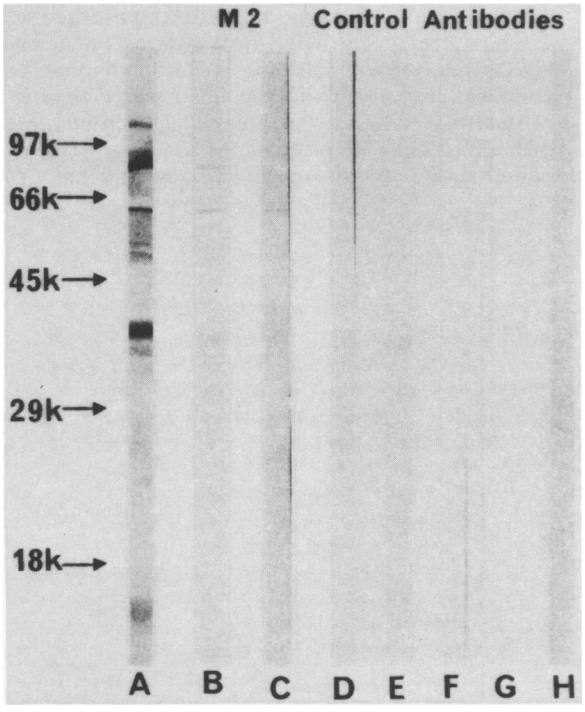

FIG. 4. Western blot analysis of antibody M2 and control monoclonal antibodies in which *K. pneumoniae* K43 was used as the antigen. Lane A, Amido black protein stain of *Klebsiella* components immobilized on a nitrocellulose membrane strip; lane B, antibody M2 probe, 0.5% Tween 20 in the washing buffer; lane C, antibody M2 probe, 1% NP-40 and 0.15 M NaCl in the washing buffer. In lanes D through H, Tween 20 was used in the washing buffer. The monoclonal antibodies used to probe the nitrocellulose membranes in lanes D through H were antibodies CBL4, CT2, 9B3, w6/32, and 66-2C9C6, respectively.

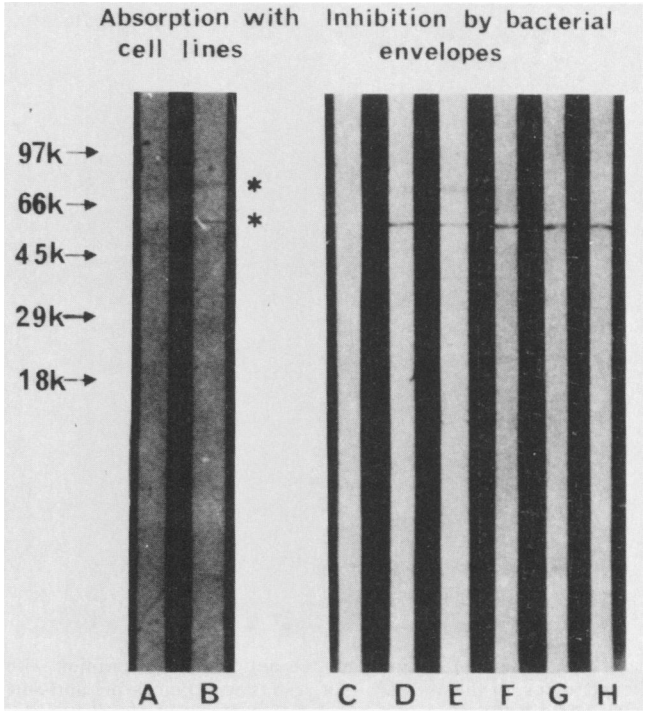

FIG. 5. Effect of addition of free antigen and absorption with lymphocytes on the Western blot results obtained with antibody M2. *K. pneumoniae* K43 components were immobilized on nitrocellulose membranes and reacted with antibody M2. In lanes A and B, the antibody was preabsorbed with HLA-B27-positive cell line HOM-2 and HLA-B27-negative cell line 8402 cells, respectively. Tween 20 (0.5%) was used in the washing buffer. The positions of the reactive antigens are indicated by asterisks. In lanes C through H, Triton X-100-solubilized bacterial envelopes were added to inhibit the Western blot reaction, and 1% NP-40 and 0.15 M NaCl were used in the washing buffer. The bacterial envelopes added were as follows: lane C, *K. pneumoniae*; lane D, *Shigella flexneri*; lane E, *Y. enterocolitica*; lane F, *Salmonella typhimurium*; lane G, *E. coli*; and lane H, *Shigella sonnei*.

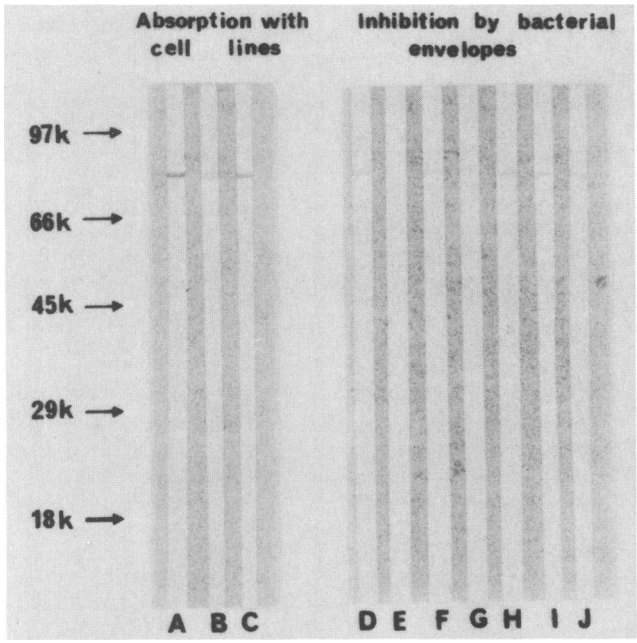

FIG. 6. Effect of addition of free antigen and absorption with lymphocytes on the Western blot results with guinea pig anti-80K antigen antibody. *K. pneumoniae* K43 components were immobilized on nitrocellulose membranes and reacted with the immune guinea pig serum. In lanes A and D, untreated serum (nonabsorbed, noninhibited) was used. In lanes B and C, the serum was preabsorbed with HLA-B27-positive cell line HOM-2 and HLA-B2-negative cell line Daudi cells, respectively. In lanes E through J, envelopes from the following bacteria were added: lane E, *K. pneumoniae*; lane F, *Shigella flexneri*; lane G, *Y. enterocolitica*; lane H, *Salmonella typhimurium*; lane I, *E. coli*; and lane J, *Shigella sonnei*.

tration of protein in each preparation was measured by the method of Lowry et al. (11). Because the concentrations were so small that they fell outside the accurate range of the standard curve for the assay, we could only estimate roughly that the total amount of each preparation was 50 to 100 μg. Each preparation was then used to immunize a separate guinea pig. The sera of the guinea pigs were then tested by using the Western blot procedure and the immunizing bacteria as the blotted antigens. Preimmune sera were negative. The serum from the guinea pig immunized with the extract from the 75K to 85K region reacted with an 80K antigen (Fig. 6, lane D). This serum was designated the anti-80K antigen serum. The serum from the guinea pig immunized with the extract from the 35K to 40K region reacted with the 39K and 37K proteins. The serum from the guinea pig immunized with the extract from the 55K to 65K region was unreactive in the Western blot assay. (The Western blot results for the latter two guinea pig sera are not shown).

Testing the cross-reactivity of the guinea pig sera by the microcytoxicity method. The guinea pig sera were tested with cell line HOM-2 cells by using the complement-dependent microcytoxicity assay. No cytotoxic activity was observed. Because of these negative results, we also tested the sera by using the Western blot and ELISA techniques.

Testing the cross-reactivity of the *Klebsiella* 80K antigen by using the immune guinea pig serum in a Western blot analysis. The effect of absorption by lymphocytes on the reactivity of guinea pig anti-80K antigen serum was tested. Decreased reactivity was observed when absorption was carried out with HLA-B27-positive cell line HOM-2, but not when absorption was carried out with HLA-B27-negative cell line Daudi (Fig. 6, lanes B and C).

Similarly, reactivity was partially blocked by adding Triton X-100-solubilized envelopes prepared from the immunizing bacterium *K. pneumoniae* K43 (Fig. 6, lane E), but not by adding envelopes prepared from the following strains of bacteria: *Shigella flexneri* 7060, *Y. enterocolitica*, *Salmonella typhimurium*, *E. coli*, and *Shigella sonnei* 7062 (Fig. 6, lanes F through J).

The results shown in Fig. 6, lanes A through C, and the results shown in Fig. 6, lanes D through J, were derived from two separate experiments. This explains why the reactivity in lane D appears to be weaker than that in lane A, although in both instances the unabsorbed antibody was tested.

Testing the cross-reactivity of the immune guinea pig serum in the ELISA. To establish preliminary conditions, we first set up an ELISA to quantitate the reactivity of the guinea pig anti-80K antigen serum. Microtiter wells are coated with Triton X-100-solubilized envelopes prepared from *K. pneumoniae* K43. Serial dilutions of the serum from $1:10^2$ to $1:10^6$ were added. The optical density reading was 1.4 at a dilution of $1:10^4$. Higher concentrations of the serum gave optical density readings of >1.5, while lower concentrations gave readings of <1.0.

Samples (100 μl) of the serum at a dilution of $1:10^4$ were then absorbed with 0.5×10^7 to 10×10^7 cells of either HLA-B27-positive cell line HOM-2 or HLA-B27-negative cell line Daudi. These absorbed sera were then tested in the ELISA. Results were expressed as percent decreases in the optical density reading compared with the unabsorbed serum sample. The decrease was much more striking when absorption was carried out with cell line HOM-2 cells than when absorption was carried out with cell line Daudi cells (Fig. 7).

As a control, we used the guinea pig serum directed against the 39K and 37K proteins for a similar analysis. A serum dilution of $1:10^4$ was used. The optical density read-

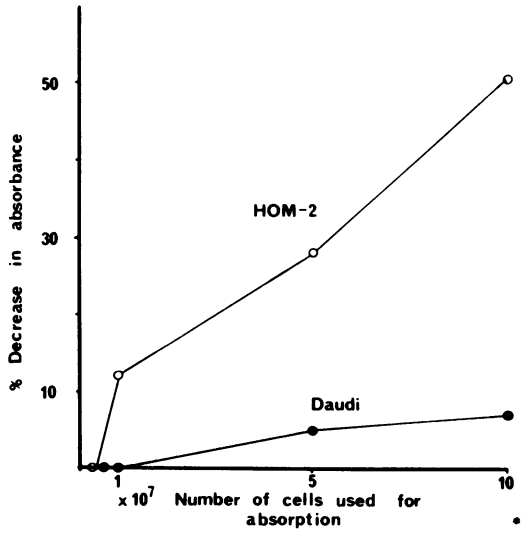

FIG. 7. Effect of absorption on the reactivity of guinea pig anti-80K antigen serum in the ELISA. Microtiter wells were coated with Triton X-100-solubilized envelopes of *K. pneumoniae* K43. Samples of the immune serum were preabsorbed with lymphoblastoid cell lines HOM-2 (HLA-B27 positive) and Daudi (HLA-B27 negative). Results are expressed as percent diseases in absorbance compared with unabsorbed serum.

ings were 1.234 and 1.252 after absorption with 10^8 cell line HOM-2 or Daudi cells. These results were very similar to the reading of 1.297 obtained by using unabsorbed serum.

DISCUSSION

In this study we addressed the questions of whether there is molecular mimicry between potentially arthritis-caused bacteria and HLA-B27 antigens and, if so, whether the cross-reactive bacterial antigens can be clearly identified. Preliminary experiments in which we used Formalin-killed bacteria and sonicated cell envelopes to absorb monoclonal anti-HLA-B27 antibodies were negative. This indicated that there are no reactive antigens on the surfaces of the bacterial envelopes. We then resorted to using Triton X-100 to solubilize the bacterial envelopes, as well as the Western blot technique. By dissociating some of the individual components of the cell envelopes from one another, we reasoned that additional antigenic determinants might be exposed.

Indeed, we found that the Triton X-100-solubilized envelopes prepared from *K. pneumoniae* K43 were able to inhibit the ELISA reactivity of one monoclonal anti-HLA-B27 antibody, designated antibody M2. The responsible cross-reactive antigens were characterized by a Western blot analysis as having apparent molecular weights of 80,000 and 60,000. Reactivity with the 60K antigen was observed, even if both 0.15 M NaCl and 1% NP-40 were used in the washing buffer. This probably indicates that the avidity of the antibody for the 60K antigen was higher than the avidity of the antibody for the 80K antigen. The specificity of the Western blot reaction was convincingly verified by several other observations. (i) Reactivity was not observed with several other monoclonal antibodies. Since generally monoclonal antibodies of the IgM isotype have more nonspecific reactions, most of the control antibodies which we selected were of this isotype. (ii) Reactivity was not observed with several other gram-negative bacteria. (iii) The reaction was diminished if monoclonal antibody M2 was first absorbed with HLA-B27-positive cells. This observation is critical for it ensures that the reactive antigens which we observed are specific for the anti-HLA-B27 antibody and not for other nautral antibacterial antibodies contaminating the ascites fluid. (iv) The reaction could be blocked if free *Klebsiella* antigens were added to the Western blot assay mixture. This finding indicates that the antibody is reactive with the 80K and 60K *Klebsiella* antigens even when they are in the fluid phase and not as artifacts of being immobilized in the solid phase on nitrocellulose membranes. (v) Finally, we isolated the 80K antigen by preparative SDS-PAGE and generated a guinea pig antiserum against it. The reactivity of this serum in the ELISA and in Western blots with *Klebsiella* envelopes was also adversely affected by absorption with an HLA-B27-positive cell line.

Collectively, the observations described above support the possibility that there is mimicry between *K. pneumoniae* K43 and HLA-B27 antigens. Using the same monoclonal anti-HLA-B27 antibody (antibody M2), van Bohemen et al. (18) were not able to detect any mimicking antigens in *K. pneumoniae*. The method which these authors used was not Western blotting. In their method, the bacterial envelopes were subjected to SDS-PAGE. The gels were frozen and then cut into consecutive segments. Each segment was then incubated, first with the monoclonal antibody and then with ^{125}I-labeled protein A. Antibody-reactive antigens were detected by autoradiography. The difference in results between the experiments of van Bohemen and our experiments was probably due to a difference in the techniques used.

Given the often-stated unreliability of the Western blot assay, our conclusion would have been more certain if the guinea pig anti-80K antigen serum which we generated had also reacted with the HLA-B27-positive cells in the microcytoxicity test. The negative cytotoxicity results which we obtained might reflect the fact that the amount of antigen available for immunization was inadequate to induce a sufficiently high serum antibody concentration. Alternatively, the cross-reactive antibody which was induced might not have been complement binding. Despite this uncertainty, the guinea pig antibody should serve as a tool to assist us in identifying the responsible envelope antigen during larger-scale isolation procedures.

In this study we did not address the question of whether the molecular mimicry phenomenon has any pathogenetic significance. It is puzzling that cross-reactivity could not be observed with *K. pneumoniae* K77 or with the arthritis-causing strain *Shigella flexneri* 7060. In addition, preliminary experiments were carried out to test anti-HLA-B27 monoclonal antibody M1. This antibody reacts with a B27 epitope which is different from the epitopes which is reactive with antibody M2. We did not observe any significant reactivity between antibody M1 and bacterial components. Nevertheless, cross-reactivity between microorganisms and host antigens has been implicated in rheumatic fever (4). It is still likely that the mimicry between arthritis-causing bacteria and HLA-B27 antigens is responsible for the development of arthritis.

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LITERATURE CITED

1. **Avakian, H., J. Welsh, A. Ebringer, and C. C. Entwistle.** 1980. Ankylosing spondylitis, HLA-B27 and *Klebsiella*. II. Cross-reactivity studies with human tissue sera. Br. J. Exp. Pathol. **61**:92–96.
2. **Brenner, M. B., S. Kobayashi, C. W. Wiesenhutter, A. Huberman, P. Bales, and D. T. Y. Yu.** 1984. In vitro T lymphocyte proliferative response to *Yersinia enterocolitica* in Reiter's syndrome. Lack of response in other HLA-B27 positive conditions. Arthritis Rheum. **27**:250–257.
3. **Calin, A.** 1984. Spondyloarthropathies: an overview, p. 1–8. *In* A. Calin (ed.), Spondyloarthopathies. Grune and Stratton, Orlando, Fla.
4. **Dale, J. B., and E. H. Beachey.** 1985. Epitopes of streptococcal M proteins shared with cardiac myosin. J. Exp. Med. **162**:583–591.
5. **Ebringer, R. W., D. R. Cawdell, P. Cowling, and A. Ebringer.** 1979. Sequential studies in ankylosing spondylitis: association of *Klebsiella pneumoniae* and active disease. Ann. Rheum. Dis. **37**:146–151.
6. **Grumet, R. C., B. M. Fendley, L. Fish, S. Foung, and E. G. Engleman.** 1982. Monoclonal antibody (B27M2) subdividing HLA-B27. Human Immunol. **5**:61–72.
7. **Kaslow, R. A., R. W. Ryder, and A. Calin.** 1979. A search for Reiter's syndrome after an outbreak of *Shigella* dysentery. J. Rheumatol. **6**:562–566.
8. **Keat, A.** 1983. Reiter's syndrome and reactive arthritis in perspective. N. Engl. J. Med. **309**:1606–1615.
9. **Kobayashi, S., M. Ogasawara, K. Maeda, and D. T. Y. Yu.** 1985. Antibodies against *Yersinia enterocolitica* in patients with Reiter's syndrome. J. Lab. Clin. Med. **105**:380–384.

9a. **Kono, D. H., M. Ogasawara, R. B. Effros, M. S. Park, R. L. Walford, and D. T. Y. Yu.** 1985. Ye-1, a monoclonal antibody that cross-reacts with HLA-B27 lymphoblastoid cell lines and arthritis-causing bacteria. Clin. Exp. Immunol. **61**:503–508.

10. **Laemmli, U. K.** 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
12. **Ogasawara, M., K. Granfors, H. H. Kono, J. L. Hill, and D. T. Y. Yu.** 1985. A *Yersinia enterocolitica* serotype O:3 lipopolysaccharide-specific monoclonal antibody reacts more strongly with bacteria cultured at room temperature than those cultured at 37°C. J. Immunol. **135**:553–559.
13. **Ogasawara, M., S. Kobayashi, S. Arai, K. Laheji, J. L. Hill, D. H. Kono, and D. T. Y. Yu.** 1985. A heat-modifiable outer membrane protein carried an antigen specific for the species *Yersinia enterocolitica* and *Yersinia pseudotuberculosis*. J. Immunol. **135**:1430–1436.
14. **Parham, P., C. J. Barnstable, and W. F. Bodmer.** 1979. Properties of an anti-HLA-A,B,C, monoclonal antibody. Use of a monoclonal antibody (w6/32) in structural studies of HLA-A,B,C antigen. J. Immunol. **123**:342–349.
15. **Seager, K., H. V. Bashir, A. F. Geczy, J. P. Edmonds, and A. deVere Tyndall.** 1979. Evidence for a specific B27-associated cell surface marker on lymphocytes of patients with ankylosing spondylitis. Nature (London) **277**:68–70.
16. **Simon, D. G., R. A. Kaslow, J. Rosenbaum, R. L. Kaye, and A. Calin.** 1981. Reiter's syndrome following epidemic shigellosis. J. Rheumatol. **8**:969–973.
17. **Towbin, H., T. Staehlin, and J. Gordon.** 1979. Electrophoretic transfer of proteins from polyacrylamide gel to nitrocellulose sheet: procedure and some applications. Proc. Natl. Acad. Sci. USA **76**:4350–4354.
18. **van Bohemen, C. G., F. C. Grumet, and H. C. Zanen.** 1984. Identification of HLA-B27M1 and M2 cross-reactive antigens in *Klebsiella*, *Shigella* and *Yersinia*. Immunology **52**:607–610.