

Structural Identity of Human Histocompatibility Leukocyte Antigen-B27 Molecules from Patients with Ankylosing Spondylitis and Normal Individuals

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ABSTRACT Although the association between human histocompatibility leukocyte antigen (HLA) B27 and ankylosing spondylitis is the prototype of HLA-disease association, the mechanism underlying these associations has not been determined. We have investigated the possibility that the B27 molecules from patients with ankylosing spondylitis are different from those of normals, and only the "different" molecules predispose the individual to disease. Biosynthetically radiolabeled HLA-B27 molecules from patients with ankylosing spondylitis and normal individuals were compared by two-dimensional gel electrophoresis and tryptic peptide mapping with high pressure liquid chromatography. Extensive charge heterogeneity in the 45,000-dalton heavy chain was detected when B27 molecules were analyzed by two-dimensional gel electrophoresis; the charge heterogeneity was reduced, but not eliminated, when the B27 molecules were treated with neuraminidase to remove sialic acid residues before analysis. No structural difference in the B27 molecules from an ankylosing spondylitis patient and a normal individual were detected by two-dimensional gel electrophoresis. Analysis of [^3H]leucine-labeled and [^3H]arginine-labeled tryptic peptides and chymotryptic peptides of the trypsin insoluble material by reverse-phase high pressure liquid chromatography revealed identity of the B27 molecules from ankylosing spondylitis patients and normal individuals. These studies indicate that development of ankylosing spondylitis in only some B27 positive individuals is not at-

tributable to those individuals possessing variant B27 molecules.

INTRODUCTION

The associations between human histocompatibility leukocyte antigen (HLA) B27 and ankylosing spondylitis is the prototype of HLA-disease association. Although HLA-B27 is found in only 8% of normal Caucasians, 90% of Caucasians with ankylosing spondylitis are B27 positive (1, 2). Likewise, HLA-B27 is present in 50% of American Blacks with ankylosing spondylitis, but in only 2% of the normal Black population (3). However, only 4% of Caucasian individuals who possess B27 develop severe ankylosing spondylitis and only 20% develop signs or symptoms of ankylosing spondylitis (4, 5). Thus, at least 80% of individuals possessing B27 never develop ankylosing spondylitis.

Despite the striking association between ankylosing spondylitis and B27, the mechanism underlying this association has not been determined. The possible mechanisms of this association can be divided into two major categories: first, the gene encoding HLA-B27 is in strong linkage disequilibrium with a disease susceptibility gene that predisposes the individual to the disease; and second, the HLA-B27 molecule directly predisposes the individual to the disease (6). There is little direct evidence supporting either of these postulates. No matter which postulate is ultimately proven correct, it also has to account for the fact that the majority of individuals with the HLA-B27 antigen never develop ankylosing spondylitis. One possible explanation is that most B27 positive individuals do not possess other unknown genetic factors and/or are not exposed to certain environmental agents necessary for the development of disease. A second possible explanation is that the B27 molecules from patients with disease are different from those of normals, and only

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the "different" molecules predispose the individual to disease.

There is evidence that suggests that differences do indeed exist in the primary structure of HLA heavy chains bearing the same private determinant. It was found that cytotoxic T lymphocytes directed against influenza virus in the context of HLA-A2 would not kill influenza-infected cells of a particular individual who was typed as HLA-A2 (7). Chemical analysis of this particular variant A2 molecule showed that it varied from the normal A2 molecules by a single tryptic peptide (8). Thus, there is precedent for serologically identical HLA molecules to differ chemically and functionally. Such differences could explain why the majority of individuals with a particular HLA antigen do not contract the associated disease.

Therefore, we have performed a series of immunochemical studies to ascertain whether there might be a structural difference between the B27 molecules present on the cells of normal individuals and those present on the cells of ankylosing spondylitis patients.

METHODS

Study subjects. Five HLA-B27 positive males with classical ankylosing spondylitis by the New York criteria (9) were studied. The five ankylosing spondylitis patients are referred to as P1-5. Seven healthy B27 positive individuals, five females and two males, served as normal controls. The normal controls were selected from laboratory personnel who had been HLA typed for other purposes. The healthy B27 positive individuals had no signs or symptoms suggestive of spondyloarthropathy and no family history of spondyloarthropathy. The normal controls are referred to as N1-7.

Serum. The anti-HLA-B27 alloserum Yarborough was kindly provided by Andrew Goldstein, University of Oregon Health Sciences Center, Portland, Oreg. This serum has been shown to be functionally monospecific for B27 by both serological and immunochemical criteria (10).

Cells. Peripheral blood cells were obtained from the study subjects by venipuncture. For the peptide maps, 100-200 ml of blood was obtained from each individual; 50 ml of blood was obtained for the two-dimensional gel electrophoresis (2-D gel)¹ studies. In collecting cells, each 100 ml of blood was immediately heparinized with 1.0 ml sodium heparin (1,000 U/ml) (Abbott Diagnostics, Diagnostic Products North Chicago, Ill.) and mixed with 40 ml 6% dextran 70 in normal saline (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) and allowed to stand for 60 min at 37°C. The lymphocyte-enriched plasma above the plasma-erythrocytes interface was collected and the lymphocytes were harvested by centrifugation.

Radiolabeling of cells and preparation of solubilized antigens. Radiolabeled antigens were prepared as described

previously (10). In brief, peripheral blood lymphocytes were resuspended at 5×10^7 cells/ml in serum-free minimal essential media deficient in leucine, methionine, or arginine. The deficient media was supplemented with [³H]leucine (150-170 Ci/mM), [¹⁴C]leucine (300 mCi/mM), [³⁵S]-methionine (900 Ci/mM) (Amersham, Corp. Arlington Heights, Ill.) or [³H]arginine (18 Ci/mM, New England Nuclear, Boston, Mass.) at a concentration of 200 μ Ci/ml and incubated for 6-8 h at 37°C in a humidified 7% CO₂ atmosphere. The cells were harvested and solubilized for 30 min at 4°C in phosphate-buffered saline containing 0.5% of the nonionic detergent Nonidet P-40 (NP-40) (Particle Data, Inc., Elmhurst, Ill.), 200 μ g/ml phenylmethylsulfonyl fluoride, 50 μ g/ml L-1-tosylamide-2-phenylethyl chloromethyl ketone (TPCK), and 50 μ g/ml N- α -p-tosyl-L-lysine chloromethyl ketone HCl (TLCK) (Sigma Chemical Co., St. Louis, Mo). Insoluble debris was removed by ultracentrifugation at 100,000 g for 60 min. The glycoprotein molecules containing glucose or mannose were then purified by binding to and elution from an affinity column of *Lens culinaris* lectin covalently bound to Sepharose 4B (Pharmacia Fine Chemicals). The purified glycoprotein fraction was incubated with 500 μ l packed protein A bearing *Staphylococcus aureus*, Cowan I strain (SaCI) per 5.0×10^8 cell equivalents of lysate for 30 min at 4°C to remove any endogenously labeled IgG. The SaCI was removed by centrifugation, and the supernate was used as a source of solubilized antigen in subsequent steps.

B27 molecules were isolated from the labeled antigen preparations by immunoprecipitation with the anti-B27 serum. For peptide maps, 15-30 $\times 10^6$ cpm of the labeled antigen preparation were incubated with 1.0-1.5 ml of anti-B27 serum for 16 h, and then incubated an additional 30 min at 4°C with 1.0-1.5 ml packed SaCI to bind the antigen-antibody complexes. For 2-D gels, 2 $\times 10^6$ cpm of [³⁵S]methionine-labeled antigen preparation, 150 μ l of anti-B27 serum, and 150 μ l SaCI were used. The antigen-antibody complexes bound to SaCI (SaCI-Ab-Ag) were pelleted by centrifuging at 1,100 g for 10 min. The SaCI-Ab-Ag pellets were washed three times with phosphate-buffered saline containing 0.25% NP-40. When antigen was isolated for analysis on 2-D gels, the SaCI-Ab-Ag pellets were eluted according to the method of O'Farrell (11). When antigen was isolated for peptide maps, radiolabeled antigen was dissociated from antibody and SaCI by boiling the pellet in 0.0625 M Tris, pH 6.8, containing 2% sodium dodecyl sulfate (SDS) and 2% 2-mercaptoethanol for 2 min, and the SaCI was removed by centrifugation. 200 μ l 2% SDS, 2% 2-mercaptoethanol, 20% glycerol, and 0.004% bromophenol blue were added and the sample was subjected to electrophoresis on 1.1 \times 20-cm cylindrical 10% polyacrylamide gels using a modification of the discontinuous SDS-polyacrylamide gel electrophoresis system originally described by Laemmli (12). The gels were cut into 2-mm slices and incubated overnight at 37°C in 1.0 ml of 0.01% SDS. An aliquot from the eluate of each gel slice Phasar liquid scintillation fluid (Amersham Corp.) was then counted in a Beckman LS8000 scintillation counter (Beckman Instruments, Inc., Palo Alto, Calif.). The fractions containing the radioactivity corresponding to the 45,000-dalton B27 heavy chains were then pooled and lyophilized. The HLA light chain, beta 2-microglobulin, migrates with the running front on a 10% gel.

Neuraminidase treatment of B27 molecules. Immunoprecipitation of B27 molecules was performed as described above. The SaCI-Ab-Ag pellets were washed twice with phosphate-buffered saline containing 0.25% NP-40. The pellets were then washed once in a solution of 0.05 M sodium

¹ Abbreviations used in this paper: 2-D gel, two-dimensional gel electrophoresis; NP-40, Nonidet P-40; SaCI, *Staphylococcus aureus*, Cowan I strain; SaCI-Ab-Ag, antigen-antibody complexes bound to SaCI; HPLC, high pressure liquid chromatography.

acetate buffer, pH 5.5, resuspended in 150 μ l of acetate buffer containing 25 U of neuraminidase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.), and incubated at 37°C for 4 h. The SaCI-Ab-Ag were then pelleted and washed once with phosphate-buffered saline containing 0.25% NP-40 before elution according to the method of O'Farrell (11).

2-D gel. 2-D gel was performed according to the method of O'Farrell (11) with some modifications. The first dimension, isoelectric focusing, was performed by using a mixture of ampholytes consisting of 1.2% (wt/vol) pH 5–7 ampholytes and 0.8% (wt/vol) pH 4–6 ampholytes (LKB Instruments, Rockville, Md.) in 5 \times 250-mm cylindrical gel tubes with a 130-mm gel. To provide an internal reference standard in each gel, so that different gels could be directly aligned, 14 C- β -lactoglobulin A (18,000 mol wt, 5.2 isoelectric point [pI]) (New England Nuclear) was mixed with each sample before the isoelectric focusing step. The samples were electrophoresed at 1.0 W/gel until the voltage reached 800 V, then under constant voltage conditions at 800 V for 18 h. After equilibration in SDS sample buffer, the isoelectric focusing gels were embedded in agarose on the top of 10% polyacrylamide slab gels prepared according to the method of Laemmli (12). 3-cm stacking gels were used to improve resolution in the second dimension. Ovalbumin, with a molecular mass of 45,000 daltons, was used as standard in the second dimension gels; a 0.5-cm piece of isoelectric focusing gel containing [14 C]ovalbumin (New England Nuclear) was also embedded on the top of each polyacrylamide slab gel adjacent to the gel containing the sample. Electrophoresis was performed at 20 mA/gel for 6 h. The slab gels were fixed in a solution containing 10% (vol/vol) glacial acetic acid and 40% (vol/vol) methanol before fluorography was performed using EN³HANCE (New England Nuclear). The dried gels were exposed to Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, N. Y.) at -70°C for 1 wk.

Preparation of enzyme digests. The lyophilized B27 45,000-dalton heavy chains eluted from the SDS-gels were redissolved and carrier bovine gamma globulin (Schwartz/Mann Div. Becton, Dickinson & Co., Orangeburg, N. Y.) was added to a concentration of 500 μ g/ml. The radiolabeled antigens were reduced with 0.15M 2-mercaptoethanol, alkylated with 0.25M twice recrystallized iodoacetamide (Sigma Chemical), and finally made 0.35M in 2-mercaptoethanol. The samples were precipitated with ice-cold 20% TCA and washed twice with 5% TCA. The precipitated material was extracted with absolute ethanol/anhydrous ether 1:1 and then with anhydrous ether alone to remove SDS and residual TCA. The ether was allowed to evaporate and the samples were resuspended in 1 ml 0.1M ammonium bicarbonate buffer (pH 8.2). TPCK-trypsin (Worthington Biochemical Corp., Freehold, N. J.) was added at a 10:1 carrier/enzyme ratio. After incubation for 1 h at 37°C, a second quantity of trypsin equal to the first was added, and incubation was continued for 16 h. Digestion was terminated by freezing the sample. The samples were lyophilized and then dissolved in 5% acetonitrile (Burdick & Jackson Laboratories Inc., Muskegon, Mich.) in 0.1% phosphoric acid (pH 2.1). The portion of the peptides soluble at this point are referred to hereafter as tryptic peptides. In some cases, the insoluble material was pelleted and redissolved in 1 ml 0.1 M ammonium bicarbonate buffer (pH 8.2). An amount of α -chymotrypsin (Worthington Biochemical Corp.) equal to two and one-half times the amount of trypsin used was then added, and the samples were incubated at 37°C for 16 h. Digestion was terminated by freezing and lyophilization and the samples were redissolved in 5% acetonitrile in 0.1%

phosphoric acid (pH 2.1). The portion of the peptides soluble at this point are referred to as the chymotryptic peptides of the trypsin insoluble material. Together the tryptic peptides and the chymotryptic peptides of the trypsin insoluble material accounted for 90% of the total radiolabel in the isolated material.

High pressure liquid chromatography (HPLC) peptide mapping. The tryptic or chymotryptic peptide digests were injected onto an E. M. Merck RP-8 column in a Spectra-Physics SP8000 high performance liquid chromatography apparatus (Spectra-Physics Inc., Santa Clara, Calif.) and eluted at 0.5 ml/min (constant flow) with a procedure modified from that of Hancock et al. (13). Injection was part of an electronically programmed chromatographic run in which the percentage of acetonitrile was increased from 5 to 50% in a linear fashion. Optical density at 206 nm (Schoeffel Instruments Div. Kratos, Inc., St. Westwood, N. J.) was monitored in line to evaluate the efficiency of digestion of the carrier protein in comparison with prior experiments. Column effluent was collected in 0.25-ml fractions in scintillation vials. After addition of scintillation fluid, radioactivity was measured using a Beckman LS-8000 counter (Beckman Instruments, Inc.).

RESULTS

2-D gel. Fig. 1 shows 2-D gel patterns of [35 S]-methionine-labeled HLA-B27 molecules from a patient with ankylosing spondylitis (P1) (Fig. 1a) and a normal individual (N1) (Fig. 1b). The corresponding portions of each gel containing the 45,000-dalton

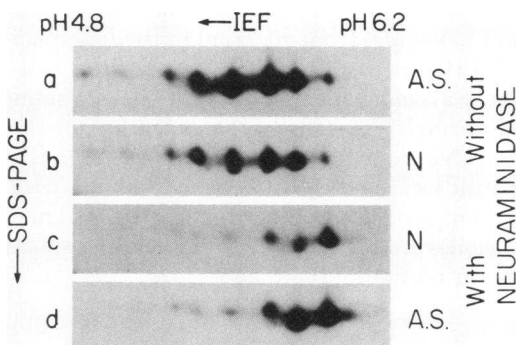


FIGURE 1 2-D gel patterns of immunoprecipitated HLA-B27 heavy chains labeled with [35 S]methionine. The corresponding portions of each gel containing the 45,000-dalton heavy chains are shown. a and d, B27 molecules from an ankylosing spondylitis patient (A.S.), b and c, B27 molecules from a normal individual (N). a and b, B27 molecules without neuraminidase treatment. c and d, B27 molecules after treatment with neuraminidase. There is extensive charge heterogeneity in the untreated B27 molecules from the two sources. There are minor differences in the pI of the two most acidic spots and the most basic spot. After treatment with neuraminidase, the basic spots have become more prominent and the acidic spots less prominent as would be expected with the removal of sialic acid residues. The input radioactivity was 20,000 cpm for each gel. The gels were exposed for 7 d. SDS-PAGE, SDS-polyacrylamide gel electrophoresis.

heavy chains are shown for close comparison. There is extensive charge heterogeneity in the B27 molecules from the two sources. The B27 molecules from the ankylosing spondylitis patient and the normal individual are very similar; each appears as eight spots, four of which are most prominent. The most acidic spot has a $pI \sim 4.9$, while the most basic spot has a $pI \sim 5.8$. Although the patterns are very similar, they are not superimposable. The two most acidic spots from the ankylosing spondylitis patient have slightly more acidic pI than the corresponding spots from the normal individual, while the most basic spot from the patient has a slightly more basic pI than the corresponding spot from the normal individual.

Neuraminidase treatment of B27 molecules. To determine how much of the observed heterogeneity was due to differences in sialylation, the immunoprecipitated B27 molecules were treated with neuraminidase to remove sialic acid residues before analysis by 2-D gels. The results are shown in Figs. 1c and 1d. After treatment with neuraminidase, the basic spots have become more prominent and the acidic spots less prominent as would be expected with the removal of sialic acid residues. The B27 molecules from both the ankylosing spondylitis patient and the normal individual now appear as six spots. Moreover, the spots in Figs. 1c and 1d are now superimposable. These results indicate that some of the charge heterogeneity of B27 molecules is due to glycosylation differences and that B27 molecules from different individuals may have small differences in their oligosaccharide moieties. When B27 molecules from additional patients (P2-4) and normals (N2-6) were analyzed by one-dimensional isoelectric focusing, small differences in the pI of some bands were noted between individuals (data not shown), but no definite pattern could be assigned to B27 molecules from patients with ankylosing spondylitis or from normal individuals.

Peptide mapping by HPLC. Tryptic peptide mapping by HPLC was utilized to investigate the primary protein structure of B27 molecules. Immunoprecipitated B27 molecules biosynthetically labeled with [3H]leucine, [3H]arginine, or [^{14}C]leucine were isolated by polyacrylamide gel electrophoresis and then eluted from the gel before digestion with trypsin. The tryptic peptides were analyzed by reverse-phase HPLC on a C8 column eluted with a linear gradient of 5-50% acetonitrile in 0.1% phosphoric acid. Valid comparison of peptide maps from different runs was possible because of the excellent reproducibility of the system, as ascertained by the bovine gamma globulin profiles monitored at 206 nm and the acetonitrile gradients. When the same sample was divided into two aliquots and run at different times, identical peptide maps were obtained.

Fig. 2 shows the tryptic peptide maps of [3H]leucine-labeled B27 molecules from a normal individual (N6, solid line) and a patient with ankylosing spondylitis (P2, dotted line). The peptide maps were run separately and then plotted on the same graph for comparison. Nine major leucine peptides are resolved in both cases. The peptide maps of the B27 leucine peptides from the ankylosing spondylitis patient and the normal individual are identical. There is a quantitative difference in the size of the peaks at fraction 110; the peak from the normal individual at this position contains 100 cpm, while the peak from the ankylosing spondylitis patient at the same position contains 20 cpm.

Only 50-75% of the total counts per minute of the tritiated amino acid-labeled tryptic peptides of B27 molecules from any individual were soluble in the HPLC starting buffer of 5% acetonitrile in 0.1% phosphoric acid. This suggests that a significant number of tryptic peptides may not be resolved in this system. To analyze a larger number of peptides from the B27

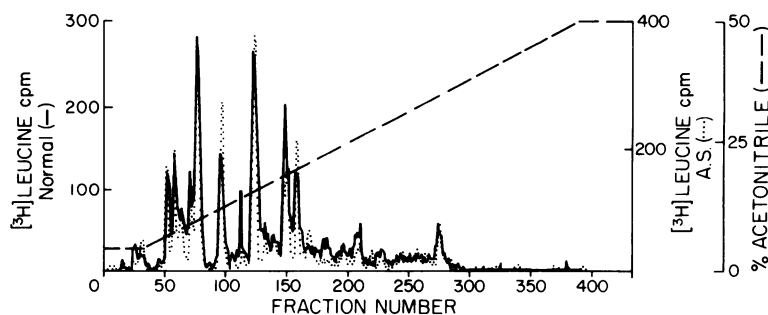


FIGURE 2 Comparative tryptic peptide maps of the [3H]leucine-labeled B27 molecules from an ankylosing spondylitis patient and a normal individual by reverse-phase HPLC. The peptide maps were run separately and then plotted on the same graph for comparison. Nine major [3H]leucine-labeled peptides are resolved in each case and are identical. The dashed line represents the acetonitrile gradient used in all the peptide maps.

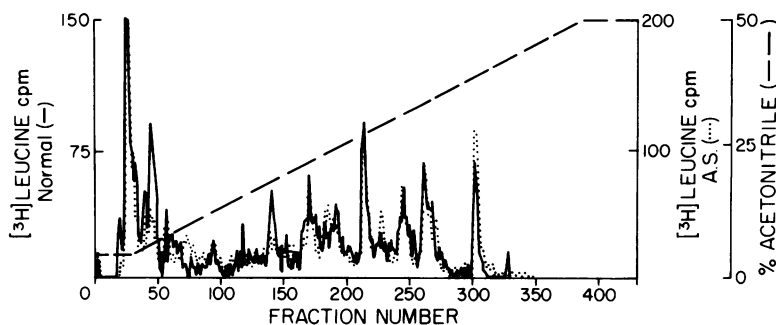


FIGURE 3 Comparison of the [^3H]leucine-labeled chymotryptic peptides of the trypsin insoluble material of HLA-B27 molecules from the same individuals as in Fig. 2. The peptide maps were run separately and then plotted on the same graph for comparison. Seven identical [^3H]leucine-labeled peptides are resolved in each case.

molecules, the pellet of trypsin insoluble material was digested with chymotrypsin before analysis by HPLC. Fig. 3 shows peptide maps of the chymotryptic peptides of the trypsin insoluble material from the same individuals shown in Fig. 2. Seven major peptides are resolved in each peptide map. Again the patterns are identical.

Fig. 4 shows the tryptic peptide maps of B27 molecules from another normal individual (N1) and another ankylosing spondylitis patient (P3). Again the peptide maps are identical; nine major leucine tryptic peptides of B27 are resolved.

To confirm the findings that the leucine-labeled peptides of B27 molecules from normal individuals and patients with ankylosing spondylitis are identical, a double-label tryptic peptide map was performed (Fig. 5). The B27 molecules from a normal individual (N1) were labeled with [^{14}C]leucine (solid line) and the B27 molecules from an ankylosing spondylitis patient (P4) were labeled with [^3H]leucine (dotted line). After the B27 molecules were isolated, they were mixed together before digestion with trypsin to eliminate possible differences in tryptic digestion, and analyzed together

by reverse-phase HPLC. Again, the peptide map profiles are identical for the B27 molecules from these two individuals.

Because of the possibility that all tryptic peptides may not be labeled with leucine, we performed additional studies on B27 molecules labeled with [^3H]arginine. Fig. 6 shows the tryptic peptide map of ^3H -arginine-labeled B27 molecules from a normal individual (N7, solid line) and a patient with ankylosing spondylitis (P5, dotted line). The peptide maps were run separately and then plotted on the same graph for comparison. 11 major arginine containing tryptic peptides are resolved in each peptide map. As with the leucine peptides, the arginine peptides are identical. The peptide maps of the chymotryptic [^3H]arginine peptides of the trypsin insoluble material are also identical; seven peptides are resolved in each peptide map (Fig. 7).

DISCUSSION

The HLA-A, B, and C antigens (including B27) are borne on molecules composed of a 45,000-dalton poly-

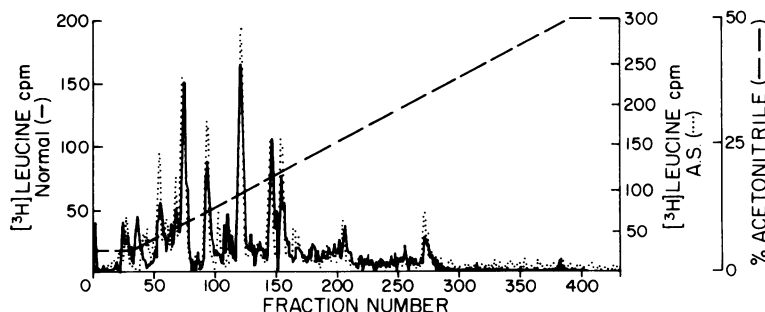


FIGURE 4 Comparative tryptic peptide maps of the [^3H]leucine-labeled B27 molecules from another ankylosing spondylitis patient and another normal individual. The peptide maps were run separately and then plotted on the same scale for comparison. The same nine major peptides are seen in each map.

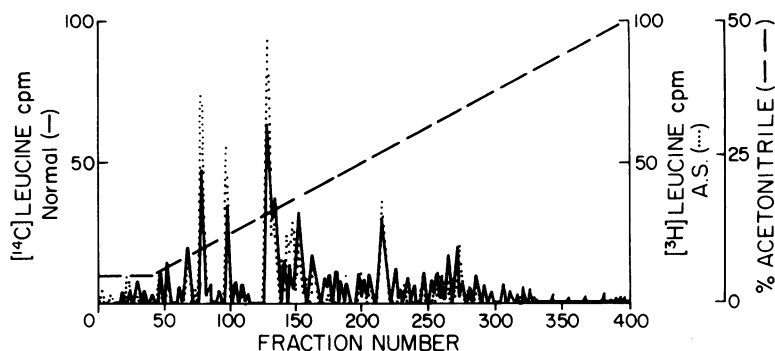


FIGURE 5 Double-label tryptic peptide maps of HLA-B27 molecules from a normal individual (labeled with [^{14}C]leucine) and a patient with ankylosing spondylitis (labeled with [^3H]leucine). The isolated B27 molecules were mixed together before digestion with trypsin and analyzed by reverse-phase HPLC. The peptide maps are identical.

morphic glycoprotein and a noncovalently linked, invariant polypeptide (beta 2-microglobulin) of 12,000 daltons. These antigens, which are expressed on virtually every human cell, are determined by the HLA-A, B, and C loci within the human major histocompatibility complex on chromosome 6. Extensive serological testing using alloantisera from multiparous females has documented the remarkable polymorphism of these antigens. The HLA-A, B, and C antigens are structurally and functionally homologous to the murine H-2 K, D, and L antigens that have been much more extensively studied (8). Extensive structural comparisons of the molecules bearing the H-2 antigens have been conducted using 2-D gel, peptide mapping and amino acid sequencing. However, similar comparative structural studies of HLA-A, B, and C antigens have been very difficult to perform. The alloantisera that are used to serologically characterize the antigens are available in only limited quantities, often have very low antibody titers, and often contain antibodies against more than one HLA specificity. Murine monoclonal antibodies have been produced against only a small number of HLA-A or B polymorphic de-

terminants (A2, A28, B17, B27) and rarely have been useful in chemical studies (14-16). Rabbit xenoantisera or murine monoclonal antibodies to monomorphic determinants on all HLA-A, B, or C molecules cannot be used to study individual specificities (17).

A large body of chemical information have been accumulated on HLA molecules isolated by classical biochemical techniques (8). However, comparative structural studies of HLA molecules have been largely limited to amino acid sequence data from HLA-B7, HLA-A2 (18), and HLA-A28 (8). The only previous comparative structural study of serologically identical HLA molecules lead to the identification of a variant HLA-A2 molecule based on different migration patterns in two-dimensional gels (7). This observation, coupled with the elegant structural and functional studies of the murine H-2 mutants (19), provided the stimulus for our comparative immunochemical studies of the B27 molecules from ankylosing spondylitis patients and normal individuals.

Attempts to perform immunochemical studies with the one available murine anti-HLA-B27 monoclonal antibody, B27m1 (16), proved unsuccessful because

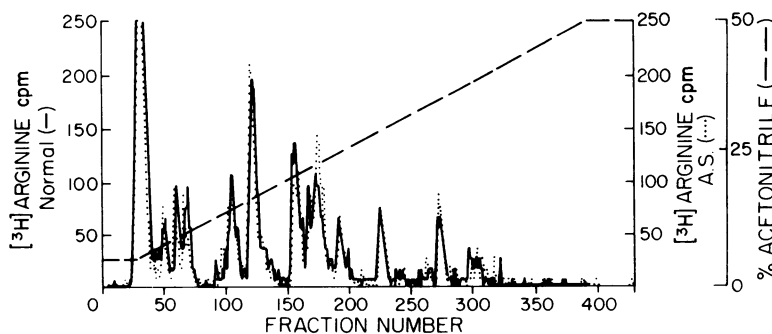


FIGURE 6 Comparative tryptic peptide maps of the [^3H]arginine-labeled B27 molecules from a normal individual and an ankylosing spondylitis patient. The peptide maps were run separately and then plotted on the same graph for comparison. The peptide maps are identical and reveal 11 major [^3H]arginine-labeled peptides.

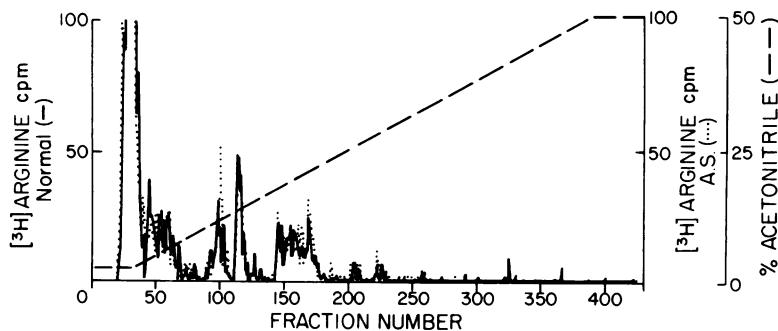


FIGURE 7 Comparison of the [^3H]arginine-labeled chymotryptic peptides of the trypsin insoluble material of HLA-B27 molecules from the same individuals as in Fig. 6. The peptide maps were run separately and then plotted on the same graph for comparison. Seven identical [^3H]arginine-labeled peptides are resolved in each case.

this monoclonal would not function adequately in immunoprecipitation assays.² We therefore performed the entire study with a human alloantiserum functionally monospecific for HLA-B27. To our knowledge, this manuscript constitutes the first report of a comparative immunochemical study in which HLA molecules were isolated using alloserum and documents the general applicability of this approach for immunochemical studies of the molecules that bear the HLA-A, B, and C antigens.

When HLA-B27 molecules were studied using 2-D gel, extensive charge heterogeneity was noted in the 45,000-dalton heavy chain. This degree of charge heterogeneity was very similar to that noted when detergent-solubilized H-2D, K, and L molecules were analyzed by the same technique (20, 21). However, less charge heterogeneity was noted when papain-solubilized HLA-A2 molecules were analyzed on 2-D gels (7). When the B27 molecules from the normal individual and the ankylosing spondylitis patient were compared, there were some minor differences noted in the isoelectric points of some of the corresponding spots. These differences were thought to be due to minor glycosylation differences between the molecules. Neuraminidase treatment of immunoprecipitated B27 molecules confirmed this postulate and produced the expected change in the 2-D gel pattern: with the removal of sialic acid residues from the carbohydrate portion of the B27 molecules, the basic bands became more prominent, the acidic bands less prominent, and the two most acidic bands were no longer present. More importantly, the 2-D gel patterns from the normal individual and the ankylosing spondylitis patient were identical after the neuraminidase treatment. By contrast, the bands of the variant HLA-A2 molecules had different pI compared with normal

HLA-A2 molecules both before and after treatment with tunicamycin to prevent glycosidation (7). However, considerable charge heterogeneity was still present in the B27 molecules even after neuraminidase treatment indicating that not all of the heterogeneity can be explained by differential glycosylation (22). This suggests that other posttranslational modifications of the B27 molecules, such as amidation or phosphorylation, may play a role in the charge heterogeneity.

Tryptic peptide mapping has been used extensively to study the murine H-2K mutants, which were initially detected because of skin graft rejection (19). For example, comparative tryptic peptide mapping of the H-2K glycoproteins from the *bm1* mutant and the parent revealed the absence of two arginine-labeled tryptic peptides and one lysine-labeled tryptic peptide from the mutant compared to the parent (23). Amino acid sequence analysis of the *bm1* mutant glycoproteins has shown that the peptide map differences are due to the substitution of two amino acids (24). In addition, the variant HLA-A2 molecule differed from normal HLA-A2 molecules by only one tryptic peptide (8). Based on this experience, any difference in the primary protein structure of B27 molecules from normal individuals and ankylosing spondylitis patients should be readily detectable with our methods. By evaluating not only the traditional tryptic peptides, but also the chymotryptic peptides of the trypsin insoluble material, we have studied at least 90% of the B27 molecule. Our analysis of HLA-B27 molecules by peptide mapping using HPLC revealed no differences between the B27 molecules isolated from ankylosing spondylitis patients and normal individuals. The [^3H]leucine-labeled tryptic peptides and the chymotryptic peptides of the trypsin insoluble material of B27 molecules from two normal individuals and two ankylosing spondylitis patients were identical. In addition, the double-label tryptic peptide maps of B27 molecules from a normal individual and an ankylosing

² Karr, R. W., Y. Hahn, and B. D. Schwartz. Unpublished observations.

spondylitis patient were identical. Finally, the peptide maps of B27 molecules labeled with [³H]arginine failed to reveal any differences between the B27 molecules from a normal individual and an ankylosing spondylitis patient. Within the limitations of our methodology, these data indicate that the primary protein structure of B27 molecules from ankylosing spondylitis patients and from normal individuals does not differ. However, because this approach does not allow analysis of 100% of the B27 molecule it is possible that subtle differences not detectable by our methods might exist.

These data do not allow one to select between the two possible mechanisms of the association of B27 and ankylosing spondylitis. Although we have shown that the primary protein structure of B27 molecules does not vary, the B27 molecule may still directly predispose an individual to the disease by a variety of mechanisms. The possibility that a disease susceptibility gene, which is in strong linkage disequilibrium with the gene encoding B27, predisposes an individual to the disease remains an attractive postulate which is supported by minimal evidence. Further studies will be required to unravel the mechanism of the association between HLA-B27 and ankylosing spondylitis.

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