Clin Exp Immunol 1993; 91:214–219

HLA-B27 subtypes in the spondarthropathies

I. L. MACLEAN, S. IQBALL, P. WOO†, A. C. S. KEAT*, R. A. HUGHES*, G. H. KINGSLEY‡ & S. C. KNIGHT *Antigen Presentation Research Group, Division of Immunological Medicine and †Division of Molecular Rheumatology, Clinical Research Centre, Harrow, *Department of Rheumatology, Westminster Hospital, and ‡Department of Medicine, Hunts House, Guy's Hospital, London, UK*

(Accepted for publication 19 October 1992)

SUMMARY

The spondarthropathy (Sp)-associated HLA-B27 antigen includes at least seven subtypes, B*2701–07, of which 01, 02, 05 and 07 occur in Caucasians. This study examined the B27 subtype distribution in British patients with Sp. The 133 HLA-B27$^+$ subjects comprised 94 European Caucasian Sp (58 ankylosing spondylitis (AS), 22 reactive arthritis (ReA; 11 sexually acquired (SARA), 11 enteric (EReA)), eight undifferentiated Sp (USp), and six pauciarticular juvenile-onset chronic arthritis (pJCA)) patients, and 34 healthy Caucasian controls, together with four Asian Indian and one Chinese. ^{35}S-labelled B27 was immunoprecipitated with anti-B27 MoAbs, and subtyped according to isoelectric point (pI) following isoelectric focussing. The use of B27 MoAb permitted subtype assignment without full class I HLA typing. The vast majority (95%) were B*2705 (Caucasian controls 31/34; AS 55/58; ReA 21/22; USp 8/8, and pJCA 6/6; Indian control 1/1 and AS 2/3; Chinese pJCA 1/1), and the remainder B*2702. No B*2701 or 07 subjects were identified. AS occurs in both B*2702 and 05 subjects, and we extend this observation to small numbers of ReA and of Indian AS subjects. This implicates molecular features shared between B27 subtypes, rather than subtype-determining regions of the antigen, in Sp pathogenesis.

Keywords Major histocompatibility complex ankylosing spondylitis reactive arthritis HLA and disease

INTRODUCTION

The class I MHC antigen HLA-B27 confers strong predisposition to the spondarthopathies ankylosing spondylitis (AS) [1,2] and reactive arthritis (ReA) [3], but the mechanism underlying the association remains unknown (reviewed in [4–7]). B27 is now known to exist in at least seven subtypes [8]. Following the Tenth International Histocompatibility Workshop (10 WS), the dominant Caucasian subtypes were designated as B*2702 and B*2705 [9]. These differ by three amino acids with a net charge difference, and in HLA-typed individuals can be discriminated by one dimensional isoelectric focussing (IEF) of immunoprecipitated, radiolabelled B27 molecules [10]. A previous study, using a combination of serological, cellular cytotoxicity, and IEF techniques found that AS patients in the Netherlands had approximately the same subtype distribution as healthy subjects [11]. We determined the B27 subtypes of HLA-B27$^+$ AS and ReA patients and of controls from a British population, using a modified immunoprecipitation protocol that did not require full class I HLA typing of subjects.

Correspondence: Dr I. L. MacLean, Division of Rheumatic Diseases, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, Texas 75235–8884, USA.

PATIENTS AND METHODS

Subjects

Patients were recruited from the Rheumatology out-patient clinics at Northwick Park, Westminster, Guys, and the Royal London Hospitals, London, UK. AS patients fulfilled the New York criteria for definite AS [12]. ReA was defined as either axial or peripheral arthropathy following a clinically documented episode of diarrhoeal illness (enteric or EReA), or of urethritis or cervicitis following sexual contact (sexually acquired ReA (SARA)). Undifferentiated spondylitis (USp) was defined as a lower limb mono- or oligoarthritis, with or without axial involvement, enthesopathy, iritis or conjunctivitis, in an individual positive for HLA-B27 and negative for IgM rheumatoid factor, but who did not fully meet the criteria for AS or ReA. Pauciarticular juvenile-onset chronic arthritis (pJCA) involved fewer than six joints at diagnosis and began before the age of 16. Patients with psoriasis or inflammatory bowel disease were specifically excluded from the study. HLA-B27 control subjects were obtained from immunology and HLA typing laboratories in the greater London region. All subjects were HLA-B27 typed using standard serological techniques. The study was approved by the Harrow District Ethical Committee (study no. EC1610).

Cell lines and monoclonal antibodies

HOM-2 is a well characterised HLA-A*0302, B*2705 homozygous line (10WS code 10W9005). An HLA-defective mutant lymphoblastoid cell line (LCL) transfected with the HLA-B*2702 gene and the neomycin resistance marker was the kind gift of Dr R. Moots, Oxford, UK, and was maintained in Geneticin (G418; GIBCO BRL/Life Technologies) 500 μg/ml before labelling. Wewak I (B*2704) was supplied by Professor A. Rickinson, Birmingham, UK. The reference lines LIH (B*2701), JL (B*2703) and 25-010 (B*2706) were supplied by Drs L. P. de Waal and J. M. Lardy, Amsterdam, The Netherlands. B*2707 (formerly B27HS) was described during this study, and was not included; its pI is identical to that of B*2701. The w6/32 [13] and B27M1 [14] hybridoma lines were obtained from Ms J. Awad, London Hospital Medical College, and ME-1 [15] from the American Type Culture Collection. ABC-m3 [16] was supplied as clarified ascitic fluid by Dr H. A. Vaughan, Melbourne, Australia.

Radiolabelling and immunoprecipitation

The method used was a modification of the 10WS IEF protocol [17]. Peripheral blood mononuclear cells (PBMC; 5×10^6) were transformed with purified phytohaemagglutinin (PHA; Wellcome) 1 mg/ml followed by recombinant human IL-2 (Glaxo) 30 U/ml and expanded in culture with IL-2 for 5–10 days before metabolic labelling with ^{35}S-methionine (ICN Biomedicals; specific activity approximately 40 TBq/mmol), 100 μCi per 10^7 viable blasts. Epstein–Barr virus-transformed B LCL were available from some control subjects and as reference lines of known B27 subtype (see above), and were processed similarly. Labelled cells (usually $5–10 \times 10^6$) were washed twice with PBS, then lysed by incubation in 1 ml precondensed lysis buffer consisting of 0·5% Triton X-114 (Sigma), 0·5% Nonidet P40 (NP40, Sigma), 1 mM phenylmethylsulphonyl fluoride, in 5 mM EDTA and 50 mM Tris, pH 7·5, for 30 min on ice. After centrifugation at 13000 ***g*** for 5 min to remove cell debris, the lysate was warmed to 37 C until turbid, centrifuged at 2000 ***g*** for 5 min, and the aqueous (upper) phase discarded. The detergent phase was diluted to 0·75 ml with a solution of 20 mM Tris, 0·5% NP40, 10 mM EDTA, and 0·1 M NaCl ('TNEN'), then precleared twice with 100 μl of a 10% suspension of formalin-fixed, Protein A-bearing *Staphylococcus aureus* Cowan strain I ('SACI'). Anti-HLA MoAb (200 μl culture supernatant; 5 μl ascitic fluid) was added for at least 30 min on a 4 C rotator, followed by 50 μl 10% SACI for at least 60 min. The SACI pellets were washed twice in an iced buffer of 0·1% SDS, 0·5% sodium deoxycholate and 1% bovine serum albumin (BSA; Cohn V, Sigma) in TNEN, and once in 0·5 M NaCl in 10% TNEN, then treated with 20 μl neuraminidase (Type VI, Sigma) 10 U/ml in 0·05 M EDTA at 37 C for 180 min, and washed once more in TNEN. In later experiments, the 10WS wash buffers and TNEN diluent were replaced with PBS containing 0·01% Tween 20 detergent (Sigma) and 2% BSA (PBS/T/BSA). The SACI were resuspended in 25 μl reducing, denaturing buffer consisting of 14 M urea, 20% v/v NP40, 5% v/v 2-mercaptoethanol, and 5% v/v ampholine 3·5–10 (Pharmacia) in water, and frozen at −20 C for up to 3 weeks before IEF.

Isoelectric focussing

Urea (electrophoresis grade, LKB) 28·5g was dissolved in a mixture of 11·5 ml water, 10 ml 10% NP40, and 8·5 ml acrylamide/bis stock solution (28·4% acrylamide, 1·6% N,N′-methylene-bis acrylamide; both Sigma), and the solution (final volume approximately 50 ml) degassed before the addition of ampholytes. In some experiments the 10WS ampholyte mixture of 1 ml Ampholines 3·5–10, 0·75 ml Ampholines 5–7 and 0·75 ml Ampholines 6–8 was modified as indicated below, and later replaced with 0·2 ml Ampholine 3·5–10 and 3 ml Pharmalyte 5–6 (all Pharmacia/LKB). The gel was polymerized with 125 μl freshly prepared 10% ammonium persulphate and 60 μl N, N, N′, N′-tetramethylethylenediamine (BDH), and cast in a $2 \times 100 \times 250$ mm glass mould. The gel was prefocussed at 10 W for 30–60 min on a watercooled LKB flatbed electrophoresis apparatus, using 230×10 mm cathode and anode filter paper (Chr 17, Whatman) strips soaked in 1 M NaOH and 1 M H_2PO_3, respectively. Samples were applied approximately 1 cm from the cathode on 10×3 mm filter paper strips, and electrofocussed at 800 V for 210 min followed by 1200 V for 30 min. Approximate pI points were established at 2·5 mm intervals along the anode–cathode axis by soaking $10 \times 2{\cdot}5$ mm gel segments in 200 μl water and measurement with a pH microelectrode. The gel was fixed with 15% acetic acid and 10% methanol in water, then soaked in Amplify (Amersham) for 30 min before drying, then autoradiography using X-ray film (Hyperfilm-MP, Amersham) and an intensifying screen. Exposure times ranged from 4 h to 2 months, but most subtyping bands were visible following 1–4 days exposure. Subtypes were assigned according to isoelectric point, in comparison with the reference cell lines. Only the HLA-B*2702 and 05 reference lines were routinely included as controls.

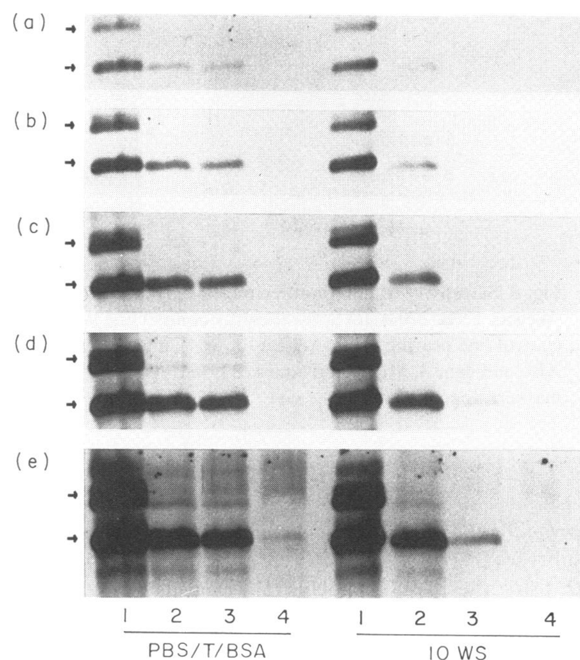

Fig. 1. Effect of buffer on immunoprecipitation of HOM-2 lysate by w6/32 (lane 1), ABC-M3 (lane 2), ME-1 (lane 3) and B27M1 (lane 4). pI markers: 5·84 (A*0302; upper arrow) and 5·66 (B*2705; lower arrow). Exposure times 20 h (a), 40 h (b), 72 h (c), 7 days (d) and 18 days (e). PBS/T/BSA, PBS with 0·01% Tween 20 and 2% bovine serum albumin; 10WS, Tenth HLA Workshop protocol diluent and wash buffers (see text and [17]).

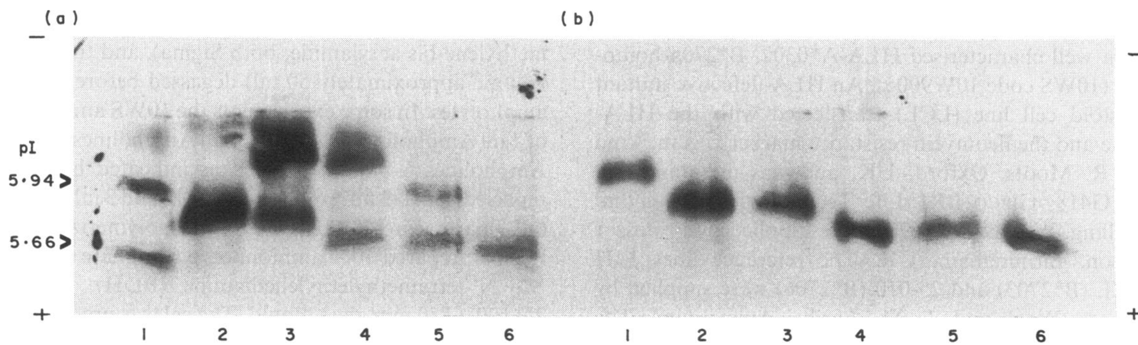

Fig. 2. Relative isoelectric focussing (IEF) positions of HLA-B27 reference cell line class I HLA immunoprecipitated by (a) w6/32 and (b) ME-1 MoAb. Lane 1, cell line 'LIH' (A24.1, 24.2; B*2701, 8); lane 2, HLA-B*2702 transfectant; lane 3, 'JL' (A23, 31; B*2703, 53); lane 4, 'Wewak 1' (A2.3, 24; B*2704, 13); lane 5, 'HOM2' (A*0302, B*2705); lane 6, '25–010' (A2; B*2706). All using PBS/T/BSA as diluent and wash buffer.

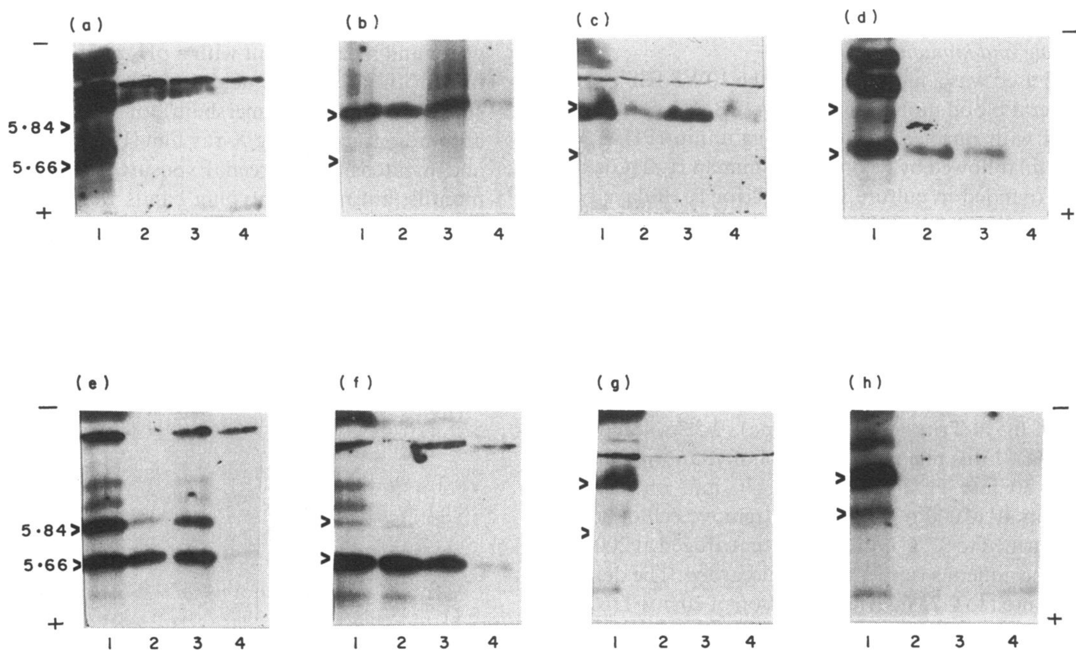

Fig. 3. Selectivity of immunoprecipitation by anti-B27. Cell lines (a) 'LIH' (B*2701), (b) B*2702 transfectant, (c) 'JL' (B*2703), (d) 'Wewak 1' (B*2704), (e) SaSw (healthy control line bearing HLA-A2, 28; B*2705, 55 (22·3)), (f) '25–010' (B*2706), (g) ArSt (healthy control line bearing HLA-A2; Bw15, w64), and (h) 1BW9 (A33; Bw65, B8). Lane 1, immunoprecipitated with w6/32 MoAb; lane 2, ABC-m3; lane 3, ME-1; and lane 4, B27M1. All using PBS/T/BSA as diluent and wash buffer. Some gels run on separate occasions; pI markers approximate.

RESULTS

Monoclonal antibodies and immunoprecipitation buffers

The subtypes B*2701 to B*2706 were all immunoprecipitated by w6/32, using the 10WS protocol. However, as w6/32 binds all class I HLA molecules and several antigens with high population frequency have a similar isoelectric point to the various B27 subtypes, interpretation of IEF patterns would require class I typing of the majority of subjects. Despite strong binding of the three B27 MoAbs (ABC-m3, ME-1 and B27M1) to B27$^+$ LCL by other methods (microlymphocytotoxicity, cellular ELISA and flow cytometry; results not included) with the 10WS protocol only ABC-m3 produced visible IEF bands, and required longer autoradiographic exposures than w6/32. Substitution of PBS/T/BSA for the 10WS diluent and wash buffers allowed use of ABC-m3 and ME-1 without significant increase in background, but B27 precipitated by B27M1 still required impractically long autoradiographic exposures (Fig. 1).

Both ABC-m3 and ME-1 precipitated B*2701 to B*2706. Using this protocol, however, discrimination of B*2704 (pI 5·68; pI values cited are those from Ref. [18]) from B*2705 (pI 5·66) was poor (Fig. 2). The selectivity of immunoprecipitation by the different MoAbs is shown in Fig. 3. Of the class I alleles potentially confused with HLA-B27 subtypes, the antigens A3, A11, A30, B8, B44, Bw64, and Bw65 were not precipitated by the anti-B27 MoAb. B55 (formerly B22.3) was precipitated by ME-1 but significantly less by ABC-m3. HLA-B*0702 was precipitated by all three anti-B27, and although its isoelectric

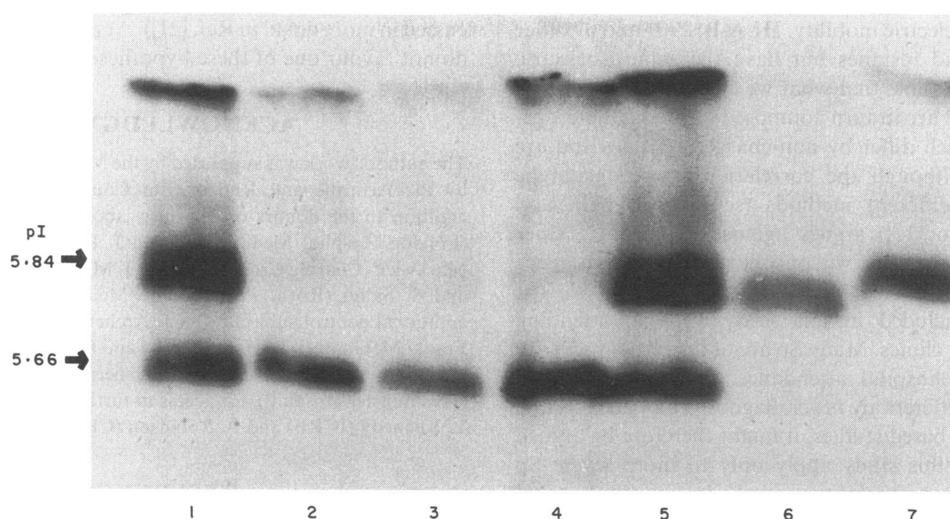

Fig. 4. Example of patient HLA-B27 subtyping gel. Lanes 1, 5, HOM-2 immunoprecipitated with w6/32; lanes 2, 3, 4, HLA-B*2705 spondylitis (Sp) patients; lane 6, HLA-B*2702 ankylosing spondylitis (AS) patient; lane 7, HLA-B*0702 control. HLA-A*0302 and B*0702 slightly more basic than B*2702 (pI 5·84, 5·84 and 5·80, respectively).

Table 1. HLA-B27 subtyping results

	HLA-B*2705 subtype	
European Caucasian	Proportion	%
Normal	31/34	91·2
SARA	10/11	90·9
EReA	11/11	100
All ReA	21/22	95·5
pJCA	6/6	100
USp	8/8	100
AS	55/58	94·8
All Sp	90/94	95·7
Indian		
Normal	1/1	100
AS	2/3	66·7

SARA, Sexually acquired reactive arthritis; EReA, enteric reactive arthritis; pJCA, pauciarticular juvenile-onset chronic arthritis; USp, undifferentiated spondorthropathy; AS, ankylosing spondylitis.

point is slightly more basic than B*2702 (pI 5·84 and 5·80 respectively), discrimination between the two was not always clear-cut (Fig. 4). Accordingly, all non-tissue typed subjects were tested for HLA-B7 by the microlymphocytotoxicity assay, to avoid the possibility of misassigning these alleles. HLA-B42 and B70.3 are uncommon alleles (Caucasian gene frequencies 0·2% and 0·3% respectively [19]), and their immunoprecipitation by the B27 MoAb was not investigated.

Subtyping

Using the short axis of the LKB 250 × 100 mm flatbed system (cathode–anode distance approximately 90 mm) and the 10WS ampholyte mixture, the HLA-B*2702 and 05 bands were separated by only 2·5 mm. Increasing the Ampholine 5–7 component to 90% of the ampholyte mixture increased this to 4·5 mm, and the further addition of 1·7% glycine as a chemical spacer (pI 6·0) to 6 mm. Using Pharmalyte 5·0–6·0 gave 9–10 mm resolution between these two predominant subtypes, and was used in subsequent experiments. Although greater separation could be achieved using the long axis (cathode–anode approximately 230 mm) overall resolution was not improved, as the bands were more blurred, had less reproduceable migration, and tended to slur. The use of 0·1 M or 0·01 M electrode solutions did not alter the pH gradient.

An example of part of a patient subtyping gel is shown in Fig. 4. w6/32 precipitates of the line HOM-2 were included on each gel, and the HLA-A*0302 (pI 5·84) and B*2705 (pI 5·66) bands used as markers. The results of the patient and control subtyping are shown in Table 1. Ninety-one per cent of European Caucasian controls and 96% of Sp patients were B*2705, and the remainder B*2702, with the excess of B*2705 in the Sp group not statistically significant ($P > 0{\cdot}10$, χ^2-test). One of three Indian AS patients had the B*2702 subtype. The single Chinese pJCA patient has been provisionally assigned as B*2705. However, as differentiation between B*2705 and B*2704 was poor using this protocol, confirmation of her subtype by other methods will be required. No B*2701 subjects were detected, in either the Sp or the healthy control group.

DISCUSSION

The interpretation of these results is subject to several reservations, regarding both the subtyping method and the selection of the subjects. Resolution between B*2704 and B*2705 (pIs 5·68 and 5·66, respectively) and between B*2702 and B*2703 (5·80, 5·77) was poor. Resolution between B*2705 and B*2706 (5·66, 5·57) was adequate (Fig. 2), but this was not formally tested by blinded subtyping. Our interpretations are therefore dependent on the previous findings that B*2704 and B*2706 are limited to Orientals, and B*2703 to Blacks. As a B*2707 control was not available it is conceivable, albeit statistically unlikely, that B*2707 subjects homozygous for B27 (HLA-B locus haplotype 2702, 2707 or 2705, 2707) may have been missed. In addition, this method can only detect subtype differences that result in

alteration in net isoelectric mobility. HLA-B*2701 and 07 differ by eight amino acid residues but have the same isoelectric mobility [8]. It is possible that what we currently recognize as B*2705 and B*2702 are in turn composed of further heterogeneous subtypes which differ by non-charged residues and are 'silent' on IEF. Although the correlation between subtype determination by different methods (serological, IEF, and cytotoxic T lymphocytes) argues against significant further heterogeneity, this possibility will only be resolved at a sequence level.

The patients included in this study were all attending hospital out-patient clinics. Many Sp are of insufficient severity to require regular hospital attendance, and indeed a high proportion of AS sufferers are never diagnosed [20]. In common with other hospital-based studies, it might therefore be argued that the results of this study apply only to more severe Sp patients. The healthy controls were mostly drawn from hospital tissue typing and academic immunology departments—hardly a random cross section of the population. However, this is unlikely to have introduced any systematic bias into the apparent subtype distribution. Some workers have reported a high incidence of undiagnosed AS in HLA-B27^{+} individuals [20]. Clinical examination and pelvic radiology were not performed on our asymptomatic 'healthy' controls, and it is conceivable that they may have included undiagnosed Sp patients. This may have helped obscure a difference in subtype incidence between patients and controls. Finally, as most Sp patients were not fully HLA typed, we do not know how many were homozygous for B27. The frequencies given therefore refer to phenotype rather than gene frequency. Within these limitations we confirm that there is no significant excess of either of the dominant HLA-B27 subtypes in AS, compared with a healthy control population, and extend this observation to ReA. Although no HLA-B*2702 EReA subjects were found, the sample size was relatively small. Similarly, in Indian AS patients the disease is not limited to HLA-B*2705; again the subject numbers are too small to allow further analysis.

Including previous studies, the data currently available indicate that Sp occurs in at least five of the known B27 subtypes (B*2701 (Choo, personal communication), 02, and 04–06). The implications of the subtyping data for the different models for the Sp-B27 association have recently been reviewed [21]. Briefly, the existence of Sp in association with multiple subtypes indicates that B27 itself (rather than an undiscovered linked 'disease susceptibility' gene [22]) is involved in disease, and argues against the significance of sequence homology between bacterial products and one B27 subtype (previously cited as evidence favouring 'molecular mimicry' in the pathogenesis of Sp [23]). Features of the HLA-B27 molecule shared between the disease-associated subtypes but unique to B27 amongst other HLA antigens, rather than the portions that determine subtype, are involved in Sp pathogenesis. These include the regions surrounding the potentially reactive unpaired sulphydryl group near the B27 epitope (cysteine residue at amino acid position 67) and the recess extending under the α_1 helix towards amino acid position 45 (the 45 or 'B' pocket [24]). These may play a critical role in the binding and presentation of processed pathogenic peptides of either autologous or microbial origin. Alternatively, they may maintain the position 67 cysteine in a suitable oxidative state for action as a receptor for exogenous products or for chemical modification to produce a neo-antigen (discussed in more detail in Ref. [21]). At present, the subtyping data do not favour one of these hypotheses over the others.

ACKNOWLEDGMENTS

The authors' work was supported by the Medical Research Council and by the Arthritis and Rheumatism Council for Research, UK. In addition to the donors of cell lines, above, we thank Drs J. Archer (London Hospital Medical College); J. Bodmer (ICRF, London); J. Markwick (Charing Cross Hospital); I. Mazek (St. George's Hospital); and S. Sidhu (Royal Postgraduate Medical School), for supplying additional control subjects; Drs J. Archer; M. Binks, A. Denman, A. Frank, M. Gumpel, R. Hermazewski and K. Williams (Northwick Park Hospital); A. Kirk (Amersham General Hospital); and A. Salih (Westminster Hospital), for access to further patient material; and Drs L. Kennedy (ICRF) and P. Robinson (CRC) for advice on IEF.

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