Significance of non-pathogenic cross reactive bowel flora in patients with ankylosing spondylitis

L E McGUIGAN, J K PRENDERGAST, A F GECZY, J P EDMONDS, AND H V BASHIR

From the Red Cross Blood Transfusion Service, Sydney, and the Rheumatology Department, St George Hospital, Sydney, Australia

SUMMARY We have previously shown that antisera raised in rabbits to certain enteric cross reactive strains of bacteria are capable of specifically lysing the peripheral blood lymphocytes of HLA-B27 positive patients with ankylosing spondylitis ($B27^+AS^+$). We now report that bacteria with cross reactive antigenic determinants are found in the bowel flora of all of 52 $B27^+AS^+$ patients but in only one of 50 HLA-B27 positive normal controls ($B27^+AS^-$). These organisms are functionally similar to the cross reactive enteric bacteria originally reported. They are not confined to a particular genus or species and their cross reactive serological nature appears to be a property shared by all enteric organisms isolated from $B27^+AS^+$ patients. Organisms with these properties have been shown to persist in the bowel flora of 14 $B27^+AS^+$ patients followed up for more than one year.

Key words: enteric bacteria, HLA-B27, lymphocytotoxicity.

The pathogenic significance of the intimate association between HLA-B27 and ankylosing spondylitis (AS)^{1,2} has been the subject of intense speculation and research for more than a decade, but the issue still remains unresolved.

One explanation for the strong association between HLA-B27 and AS is that the HLA-B27 allele is in linkage disequilibrium with a 'disease susceptibility gene' for AS (the 'two gene' theory).³ According to this view HLA-B27 is simply a marker and therefore plays no significant part in the pathogenesis of the disease. The discordance for AS in identical twins⁴ and the failure of family and population studies to identify the independent segregation of AS and HLA-B27 (except in those families where conditions associated with AS, such as inflammatory bowel disease or psoriasis, also occur) have cast serious doubt on this theory.⁵

Alternatively, it has been proposed that HLA-B27 itself is the predisposing element in AS. This idea, known as the 'one gene theory', implies an interaction between an environmental stimulus and

the HLA-B27 antigen which initiates a sequence of events leading to AS.⁶ The association of HLA-B27 with Reiter's syndrome and other reactive arthritides,^{7,8} known to be triggered by micro-organisms, has highlighted the possibility that the environmental stimulus interacting with HLA-B27 could also be of microbial origin.

We have previously investigated the possibility of an interaction between bacteria and HLA-B27, and we have shown that antisera raised in rabbits to certain strains of enteric bacteria are able to lyse specifically the peripheral blood lymphocytes (PBL) of the majority of HLA-B27 positive patients with AS ($B27^+AS^+$) but not those of HLA-B27 negative spondylitics ($B27^-AS^+$) or HLA-B27 positive or negative normals ($B27^+AS^-$ and $B27^-AS^-$ respectively).⁹

Further, we have been able to show that the culture filtrates of these specific cross reactive strains of bacteria contain a factor which is capable of 'modifying' $B27^+AS^-$ PBL so that they become susceptible to lysis by our antisera (i.e., they were serologically similar to $B27^+AS^+$ PBL).¹⁰

In an earlier publication cross reactive organisms were reported to be present in the bowel flora of all 20 $B27^+AS^+$ patients tested.¹¹ We have now studied

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Correspondence to Dr L McGuigan, Red Cross Blood Transfusion Service, 153 Clarence Street, Sydney, NSW 2000, Australia.

another 52 B27⁺AS⁺ patients and compared them with 50 B27⁺AS⁻ normal blood donors. Cross reactive bowel organisms were detected in only one B27⁺AS⁻ normal donor but in all B27⁺AS⁺ patients, where they have been shown to persist for long periods. In addition, the culture filtrates of cross reactive bacteria are able to modify B27⁺AS⁻ cells, while antisera raised to these organisms specifically lyse B27⁺AS⁺ cells. These findings strongly suggest a pathogenetic role for these organisms in ankylosing spondylitis.

Patients and methods

PATIENTS AND CONTROLS

Fifty two B27⁺AS⁺ patients were selected for study. The criteria used for selection were the patients' availability for participation in the study, the presence of the HLA-B27 tissue type, and unequivocal radiological and clinical evidence of AS. A relevant medical history and physical examination were performed on each patient. The range of neck and back movements was noted by standardised techniques, and these were compared with previously established values for age and sex matched controls.^{12 13} All 52 patients satisfied New York criteria for AS.¹⁴

Fifty controls were selected from the normal HLA-B27 positive blood donors attending the Red Cross Blood Transfusion Service. The only criterion

for selection, apart from the presence of HLA-B27 antigen, was the individual's willingness to participate in the study. Of the 53 contacted, only three were unable to take part in the study.

A relevant medical history, including those historical features pertinent to AS, was recorded.¹⁵ The range of neck and back movements was elicited in the same manner as for the AS patients. Further details of the characteristics of the B27⁺AS⁺ and B27⁺AS⁻ groups are shown in Table 1.

SEPARATION OF LYMPHOCYTES

Fifty millilitres of venous blood were obtained from each patient and control. Peripheral blood lymphocytes (PBL) were separated from heparinised blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) for 25 min at 580 g. The leucocyte band was removed and washed with RPMI 1640 medium (Flow Laboratories, Stanmore, New South Wales, Australia) containing 2 ml of heparin (1000 U/ml), 10 ml of 1 M HEPES (N-2-hydroxyethylpiperazine-N¹-2-ethanesulphonic acid; Sigma Chemical Co.) buffer, 5 ml of L-glutamine (200 mM), and 2 ml of penicillin-streptomycin (500 IU and 5000 µg/ml; Flow Laboratories) per 500 ml of RPMI 1640 medium. The leucocytes were suspended in RPMI 1640 medium containing 10% human group A serum, centrifuged for a further 10 min at 180 g, and adjusted to a concentration of 1×10^5 – 2×10^5 cells/100 µl of medium before use in a lymphocytotoxicity assay. If the cells were not used immediately they were resuspended in an equal volume of 20% dimethylsulphoxide in RPMI 1640 medium, frozen in a controlled freezing cycle apparatus to -100°C, and then stored in liquid nitrogen.

TISSUE TYPING

HLA-B27 tissue typing was performed by the standard two stage Terasaki technique.¹⁶

LYMPHOCYTOTOXICITY ASSAY

This assay has been previously described in detail.⁹ Briefly, PBL from B27⁺AS⁺ patients and B27⁺AS⁻ controls were suspended at a concentration of 2×10^6 cells/ml and labelled with 100 µCi of ⁵¹Cr (specific activity 200–500 µCi/µg; Amersham). After washing, 100 µl of the ⁵¹Cr labelled cell suspension was added to 10 × 75 mm plastic tubes and incubated with 100 µl of antiserum at room temperature (20–28°C) for 30 min. Rabbit complement (100 µl) (Pel-Freez Biologicals, Arkansas, USA) was then added to each tube and the incubation continued for a further 60 min. The cells were centrifuged at 2000 rpm for 15 min, and 100 µl of the supernatant was counted in a Beckman auto gamma scintillation spectrometer.

Table 1 Clinical characteristics of B27⁺AS⁺ patients and B27⁺AS⁻ controls

	HLA-B27 positive ankylosing spondylitis	Normal HLA-B27 positive blood donors
Number	52	50
Sex	46M, 6F	37M, 13F
Age (years)		
Mean	45.7	40.1
Range	26–77	19–65
Disease duration (years)		
Mean	20.8	—
Range	5–53	—
Number with moderate to severe restriction of neck movements	42	1
Number with restricted back movement in three planes	41	0
Number with chest expansion <2.5 cm	36	0
Number with chronic thoracolumbar back pain	51	5

The amount of radioactivity in the test samples was compared with the radioactivity in an equal volume of the supernatant from tubes containing cells in medium plus complement only, and that present in tubes containing PBL lysed by the detergent Nonidet P40. The results were expressed as the percentage of maximum ^{51}Cr released, which was calculated as follows:

$$^{51}\text{Cr release (\%)} = 100 \times \frac{\text{radioactivity released by antiserum} - \text{radioactivity released in the absence of antiserum}}{\text{radioactivity released by Nonidet P40 solubilised cells} - \text{radioactivity released in the absence of antiserum}}$$

BACTERIAL CULTURES

Faecal swabs were obtained from patients and controls and cultured on MacConkey agar. An isolate of *Escherichia coli* was selected as representative of normal bowel flora. We have previously established that this is a valid assumption¹¹ for the purposes of our absorption technique on the basis of absorbing five isolates of enteric bacteria from each of 10 B27⁺AS⁺ patients and 10 B27⁺AS⁻ controls. Either all five isolates from each patient or control carried the cross reactive determinant or none of the five isolates carried this determinant.

Aerobic cultures of each bowel isolate were grown in Luria broth (50 ml) for 12–15 h at 37°C on a rotary shaker. Cultures were harvested for centrifugation at 2000 g for 20 min. Bacteria were suspended in 5 ml of the Luria broth and killed by the addition of 1 ml of 10% formaldehyde. The formalin killed bacteria (10^8 – 10^9 /ml) were washed twice with sterile 0.85% NaCl and used in the absorption assay.

BACTERIAL IDENTIFICATION

The identification of bowel isolates was performed by a standard reference laboratory (Microbiology Department, St George Hospital, Sydney).

PREPARATION OF ANTISERA

Preparation of bacterial cultures for the production of cross reactive antibacterial sera was carried out as previously described.¹¹ It should be noted that cross reactive antibacterial sera refer to antisera against previously characterised cross reactive organisms such as *Klebsiella* sp K43 BTS 1, *Salmonella typhimurium* BTS 69, and *Shigella boydii* BTS 20.¹⁷ Since antisera to these enteric organisms all react specifically with a determinant on B27⁺AS⁺ cells, as measured by lymphocytotoxicity, it was considered valid to use these antisera interchangeably.

ABSORPTION TECHNIQUE

Three hundred microlitres of an antiserum (which gave a ^{51}Cr release value of greater than 50% when tested on B27⁺AS⁺ PBL) was used for absorption with about 10^9 bacteria prepared as outlined above. After incubation with the bacteria for two hours the antiserum was removed by centrifugation and reabsorbed with a further 10^9 bacteria of the same isolate for a further two hours. The antiserum was again separated from bacteria by centrifugation and then reacted with B27⁺AS⁺ PBL in a ^{51}Cr release assay. If after absorption the antiserum still produced a ^{51}Cr release value of >50% then the bacteria had failed to remove the lymphocytotoxic activity of the antiserum, but if the cytotoxicity was reduced to <10% the activity of the antiserum was considered to have been absorbed by the bowel isolate of that patient.

MODIFICATION ASSAY

PBL from B27⁺AS⁻ individuals that had been shown to give low levels of cytotoxicity (when tested with antisera to cross reactive organisms in the lymphocytotoxicity assay) were used as targets in the lymphocytotoxicity assay after modification by bacterial culture filtrates as previously described.¹⁰ Briefly, about 5×10^6 PBL were incubated overnight at 37°C in an atmosphere of 5% CO₂ in air in 1.8 ml of RPMI 1640 medium containing 20% human group A serum and 200 µl of bacterial culture filtrate. The cells were then centrifuged at 800 g for 10 min, resuspended in 1 ml of RPMI 1640 containing 20% group A serum, labelled with ^{51}Cr , and after two washings used in the lymphocytotoxicity assay.

Results

LYMPHOCYTOTOXICITY

Lymphocytotoxicity assays were performed on the PBL of each patient and normal control (Table 2). We have previously reported that we considered lymphocytotoxicity values of >50% as positive, while a value of <20% was considered negative.¹⁷ On this basis all B27⁺AS⁺ patients investigated in this study were positive.

BACTERIAL IDENTIFICATION

The bowel isolates from 14 patients and 14 controls were microbiologically identified. All were *E coli* except for one isolate of *Proteus vulgaris* from a B27⁺AS⁺ patient. The remaining bowel isolates from patients and controls were not formally identified as we have previously shown that if a cross reactive organism is found in the bowel flora many other species of enteric bacteria within that indi-

Table 2 Summary of the numbers of B27⁺AS⁺ patients and B27⁺AS⁻ controls that (i) had PBL lysed by anti-cross-reactive bacterial antisera and (ii) harboured cross reactive organisms in their bowel flora

	Patient category	
	B27 ⁺ AS ⁺	B27 ⁺ AS ⁻
Number	52	50
Number with PBL ⁵¹ Cr release values >50% (i.e., positive)	52	0
Number with PBL ⁵¹ Cr release values <20% (i.e., negative)	0	50
Number with bowel isolates capable of absorbing antisera to cross reactive organisms	52	1

vidual's bowel flora will also carry the cross reactive determinant.¹¹

ABSORPTION OF ANTISERUM

The results of the absorption studies are also shown in Table 2. All 52 B27⁺AS⁺ individuals had bowel isolates capable of absorbing the activity of cross reactive antisera, but only one of the B27⁺AS⁻ controls harboured such organisms.

To establish that these bowel isolates were specifically absorbing antibodies directed to cross reactive organisms and not to the HLA-B27 specificity we absorbed anti-HLA-B27 tissue typing sera with isolates from five individuals carrying cross reactive organisms and with five other isolates identified as being non-cross reactive on the basis of the absorption technique. The anti-HLA-B27 serum was then tested in the complement mediated ⁵¹Cr release

cytotoxicity assay with B27⁺AS⁺ PBL as targets. None of the 10 bowel isolates was able to absorb the anti-HLA-B27 activity of alloantisera, indicating that the bowel isolates of the 52 B27⁺AS⁺ patients and one B27⁺AS⁻ control were indeed related to the cross reactive bacteria against which our rabbit antisera are directed.

MODIFICATION OF B27⁺AS⁻ PBL BY CROSS REACTIVE AND NON-CROSS REACTIVE BACTERIAL CULTURE FILTRATES

To establish that the organisms identified as being cross reactive on the basis of the absorption studies were functionally similar to those previously described we tested the culture filtrates of five cross reactive and five non-cross reactive organisms for their ability to modify B27⁺AS⁻ PBL. Filtrates from cross reactive bowel isolates caused B27⁺AS⁻ PBL to become susceptible to lysis (Fig 1A) by antibacterial sera, whereas filtrates from non-cross reactive bowel isolates had virtually no effect (Fig. 1B).

ANTISERA TO CROSS REACTIVE AND NON-CROSS REACTIVE BOWEL ISOLATES

To demonstrate that the cross reactive organisms identified by the absorption test were capable of producing specific antisera, two cross reactive *E coli* bowel isolates (designated A and B) and one non-cross reactive *E coli* bowel isolate (designated C) were injected into three separate rabbits and boosted twice as previously described.⁹ The specificity of the resultant antisera is shown in Table 3. The antisera raised to the cross reactive organisms A and B were highly specific for B27⁺AS⁺ PBL, while those raised to the non-cross reactive isolate C failed to lyse B27⁺AS⁺ cells.

Fig. 1 Effect of incubation of B27⁺AS⁻ PBL (shown in black circles) with the culture supernatants of cross reactive organisms from five B27⁺AS⁺ patients (A) and non-cross reactive organisms from five B27⁺AS⁻ controls (B). Before modification the B27⁺AS⁻ PBL are not susceptible to lysis by antisera to cross reactive organisms. Each of the five supernatants from the bowel isolates of the five B27⁺AS⁺ patients was able to modify these PBL so that they became susceptible to lysis (A) but none of the five supernatants from the bowel isolates of the five controls was able to do this (B).

Table 3 ⁵¹Cr release in the complement mediated lymphocytotoxicity assay using antisera raised to cross reactive E coli bowel flora isolates A and B and non-cross reactive E coli bowel flora isolate C*

	Category of patient or control			
	B27 ⁺ AS ⁺	B27 ⁺ AS ⁻	B27 ⁻ AS ⁺	B27 ⁻ AS ⁻
Reactivity with antiserum to cross reactive E coli (A)				
No of patients with ⁵¹ Cr > 50%	32	0	0	0
No of patients with ⁵¹ Cr < 20%	2	55	3	35
Total number of patients tested	34	55	3	35
Reactivity with antiserum to cross reactive E coli (B)				
No of patients with ⁵¹ Cr > 50%	6	0	0	0
No of patients with ⁵¹ Cr < 20%	0	5	3	6
Total number of patients tested	6	5	3	6
Reactivity with antiserum to non-cross reactive E coli (C)				
No of patients with ⁵¹ Cr > 50%	0	0	0	0
No of patients with ⁵¹ Cr < 20%	34	55	3	35
Total number of patients tested	34	55	3	35

*5×10⁶ PBL from each patient or control in each category were used in each lymphocytotoxicity assay. Values of ⁵¹Cr release > 50% of total release were considered positive and values of ⁵¹Cr release < 20% of total release were considered negative.

PERSISTENCE OF CROSS REACTIVE BOWEL ISOLATES IN B27⁺ AS⁺ PATIENTS

We have repeated the isolation and absorption of bacteria from the rectal swabs of 14 B27⁺ AS⁺ patients one year after the cross reactive organisms were initially detected. All 14 patients at follow up had enteric organisms with cross reactive antigenic determinants.

Discussion

We have previously shown that antisera raised in rabbits to a number of enteric organisms are able specifically to recognise and lyse the PBL of B27⁺ AS⁺ patients in a ⁵¹Cr release complement mediated cytotoxicity assay.⁹ Although this finding highlighted a possible association between these cross reactive organisms and AS, the clinical relevance of these organisms was unknown. We have shown in this study that organisms with cross reactive antigenic determinants can be found in the bowel flora of 100% of B27⁺ AS⁺ patients but in only 2% of B27⁺ AS⁻ controls. We have also shown that these bowel isolates are antigenically and functionally similar to the cross reactive enteric bacteria previously reported.⁹ This conclusion is based firstly, on the ability of these bowel isolates to elicit in rabbits antibodies (Table 3) of the same specificity as the cross reactive organisms described in our earlier work and secondly, on the modifying factor activity of their culture filtrates (Fig. 1).

In an earlier study we showed that the enteric

organisms identified by our absorption technique were not confined to a particular genus or species.¹¹ In addition, we established, by performing absorptions with isolates from a number of B27⁺ AS⁺ patients and B27⁻ AS⁻ and B27⁺ AS⁻ normal controls, that if a cross reactive bacterium is detected in a randomly selected bowel isolate then this property is likely to be shared by all other isolates from a particular patient.¹¹ It would seem therefore that we are detecting a property common to all bowel flora in B27⁺ AS⁺ patients rather than a marker or determinant restricted to a particular species. Once present in the bowel flora these cross reactive organisms appear to persist for long periods (i.e., at least one year). In fact, although our follow up data are limited to 14 patients, we have not observed any instance where these cross reactive organisms were lost from the bowel flora. This finding, in addition to the observation that we were able to find these organisms in all B27⁺ AS⁺ patients, would suggest that the presence of these cross reactive organisms is independent of the activity of AS. It would also appear that there is no evidence of molecular mimicry between the cross reactive determinant and the HLA-B27 antigen as these cross reactive organisms were not able to absorb alloantisera against HLA-B27.

Some might suggest that the presence of these organisms in B27⁺ AS⁺ is an epiphenomenon related to the disease or its treatment rather than a reflection of a possible causal relationship. Neither possibility is likely as the organisms were found in

two normal individuals (one in this study and one in a previous study¹¹), and the presence of the organisms in the AS patients was not related to their intake of anti-inflammatory medications. If these organisms are not an epiphenomenon what role, if any, do they have in the pathogenesis of AS?

We have previously suggested and produced evidence that is consistent with the hypothesis that the cross reactive determinant on these bacteria is coded by an extrachromosomal element, possibly a plasmid. It has been shown in a preliminary study that the production of the cross reactive determinant can be eliminated by curing the bacteria of plasmids with acridine orange. In a transconjugation experiment a plasmid free *E coli* strain acquired the ability to express the cross reactive determinant after the transfer of plasmids from a cross reactive strain of *Klebsiella*.¹⁸ This demonstration that the gene coding for the cross reactive factor resides on a mobile genetic element in certain bacteria raises interesting questions with regard to the expression of this factor by B27⁺AS⁺ cells. Firstly, could it be that the presence of the HLA-B27 antigen in some way creates a suitable environment for these cross reactive organisms to colonise the bowel flora of HLA-B27 positive hosts and secondly, could these organisms transfer genetic material, coding for the cross reactive determinant, to the other bowel flora and perhaps even to HLA-B27 positive cells of the host?

If this were the case then one should be able to find among the relatives of B27⁺AS⁺ probands a cohort of young B27⁺AS⁻ individuals who harbour the cross reactive organisms but have no symptoms of AS. Moreover, with the use of appropriate molecular genetic techniques one should be able to detect the transfer of the bacterial deoxyribonucleic acid (DNA) to human cells. Our group is currently investigating both these possibilities.

If our postulate on the transfer of specific genetic material is correct then several important questions on the acquisition of prokaryotic DNA by HLA-B27 positive cells still remain to be answered. For example, if this genetic material is plasmid borne then how is it able to enter so many species of bacteria and indeed, how is it capable of gaining entry into human cells? Even if these two issues can be resolved we still must identify the mechanism whereby the acquisition of the cross reactive factor leads to the manifestations of AS. At present we are unable to answer these questions or delineate the pathogenetic mechanisms of AS, but we feel justified in pursuing our hypothesis because it appears to be the most rational and fruitful approach in explaining our findings to date. If and

when answers to these questions become available the implications will be enormous, not only for AS but probably for other HLA linked diseases which may be triggered by environmental agents.

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