INFECTION AND IMMUNITY, Dec. 1984, p. 686–689
0019-9567/84/120686-04$02.00/0

Vol. 46, No. 3

Persistence of HLA-B27 Cross-Reactive Bacteria in Bowel Flora of Patients with Ankylosing Spondylitis

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Received 27 July 1984/Accepted 5 September 1984

Previous studies have shown that antisera raised in rabbits to certain enteric bacteria (cross-reactive bacteria) are capable of specifically lysing in a ^{51}chromium-release lymphocytotoxicity test the lymphocytes of HLA-B27-positive ($B27^+$) patients with ankylosing spondylitis (AS). This study investigated the clinical relevance of this finding by ascertaining whether *Escherichia coli* isolated from the rectal swabs of 20 $B27^+$ AS patients ($B27^+AS^+$) and 46 controls (35 $B27^-AS^-$ and 11 $B27^+AS^-$) were able to absorb the lymphocytotoxic activity of these antisera. All isolates from $B27^+AS^+$ patients and one from a $B27^-AS^-$ individual were capable of removing this activity. These organisms persisted in the bowel flora of five selected patients for at least 9 months. Cross-reactive bacteria were also found in a range of gram-positive organisms, including streptococcal, staphylococcal, and clostridial species. The relevance of these findings is discussed in terms of a pathogenetic concept relating the persistence of cross-reactive bacteria in the bowel flora of $B27^+$ individuals to an early event in the development of AS.

The concept of an environmental trigger in the aetiology of several seronegative arthropathies has been the subject of intense debate. Apart from the undisputed importance of the tissue antigen HLA-B27 (B27), the pathogenic relevance of prior gastrointestinal infections in these diseases has remained uncertain. Although epidemiological studies indicate a temporal relationship between infection and the onset of the clinical features of reactive arthritis or Reiter's syndrome (1, 5, 8, 10), evidence of such an association in ankylosing spondylitis (AS) is tenuous. Previous studies from our laboratory have established that a range of pathogenic enteric bacteria shares an outer membrane determinant (cross-reacts) with the B27-positive tissues ($B27^+AS^+$) of AS patients. Specifically, antisera to these enteric organisms lyse the lymphocytes of $B27^+$ patients with AS but fail to lyse the cells of $B27^+$ and $B27^-$ clinically normal individuals (7, 12). The implication of these findings is that these organisms may represent an "infectious" episode, and therefore, their involvement may be an important early event in the pathogenesis of AS. To strengthen the argument for a role for bacteria in this disease, we now report that the bowel flora of AS patients contain nonpathogenic cross-reactive bacteria. Moreover, cross-reactivity with $B27^+AS^+$ cells is not restricted to gram-negative enteric organisms but is readily detectable in a range of gram-positive bacteria. These findings are discussed in terms of a possible in vivo pathogenetic model involving a widespread, readily transmissible genetic element(s).

MATERIALS AND METHODS

Patients. The HLA-$B27^+$ patients were suffering from classical AS as defined by the New York criteria (2). HLA-$B27^+$ and HLA-$B27^-$ healthy controls were members of staff or blood donors with no history of joint disease. All patients and controls were tissue typed as described previously (9).

Bacterial cultures. Rectal swabs were obtained from patients and controls and cultured for *Klebsiella* sp. and *Escherichia coli* on MacConkey agar (Oxoid Ltd., London, United Kingdom). Streptococci and staphylococci were selected after anaerobic and aerobic incubation on horse blood agar plates (Media Makers, Sydney, New South Wales, Australia). Clostridia were selected anaerobically on TSC agar (Oxoid) and *Clostridium perfringens* was isolated by positive confirmation on Nagler plates. The identity of isolates was confirmed by a standard reference laboratory (Microbiology Department, Institute of Clinical Pathology and Medical Research, Westmead Centre, Parramatta Hospital, Sydney, Australia).

Growth and harvesting of bacteria. Before absorption studies, aerobic cultures were grown in Luria or nutrient broth (50 ml) for 12 to 20 h at 37°C on a rotary shaker. Anaerobic cultures were grown in thioglycolate medium. Cultures were harvested by centrifugation at 20,000 × *g* for 20 min. Bacteria were suspened in 10 ml of the appropriate broth and then killed by adding 1 ml of 10% formaldehyde. The Formalin-killed bacteria (10^8 to 10^9/ml) were washed twice with sterile 0.85% NaCl and either used immediately for absorption studies or stored at −30°C until required.

Preparation of antisera. Preparation and absorption of bacterial cultures with cross-reactive antibacterial sera were carried out as described previously (3, 14). It should be noted that cross-reactive antibacterial sera refer to antisera raised against previously characterized cross-reactive organisms such as *Klebsiella* sp. K43 BTS1, *Salmonella typhimurium* BTS 69, and *Shigella boydii* BTS 20 (12). Since antisera to these enteric organisms react specifically with a common determinant on $B27^+AS^+$ cells as measured by lymphocytotoxicity, it was considered valid to use these antisera interchangeably.

Separation of lymphocytes. Peripheral blood lymphocytes were separated from heparinized blood by centrifugation on Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) for 20 min at 580 × *g*. The leukocyte band was removed and washed with RPMI 1640 (Flow Laboratories, Stanmore, New South Wales, Australia) medium containing 2 ml of heparin (1,000 U/ml) 10 ml of IM HEPES (*N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid; Sigma Chemical Co.)

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TABLE 1. Bacteria from rectal swabs of $B27^+AS^+$ patients and a clinically normal individual removing the lymphocytotoxic activity from cross-reactive antibacterial sera

Bacteria	Target lymphocytes $B27^+AS^+$
None	62–95[a]
Klebsiella sp. K43 BTS1[b]	0–6
S. typhimurium BTS 69[b]	0–8
Klebsiella sp. F77[d]	65–84
Rectal swab isolates of *E. coli* from:	
20 of 20 $B27^+AS^+$ patients	0–5
11 of 11 $B27^+AS^-$ controls	68–92
1 of 35 $B27^-AS^-$ controls[c]	0–4
34 of 35 $B27^-AS^-$ controls	74–90

[a] Percentage of maximum ^{51}Cr released measuring absorption of cross-reactive antibacterial sera. The range (percent lysis) refers to results obtained with target cells used in a series of assays. It was not possible to test all patients and controls at the same time.

[b] Cross-reactive organisms as previously described (12).

[c] *E. coli* from the bowel flora of this individual has been tested previously (12).

[d] A "non-cross-reactive" isolate which does not cross-react with $B27^+AS^+$ cells (12).

buffer, 5 ml of L-glutamine (200 mM), and 2 ml of penicillin-streptomycin (500 IU and 5,000 μg/ml; Flow Laboratories) per 500 ml of RPMI medium. The leukocytes were suspended in RPMI medium containing 10% human A serum, centrifuged for a further 10 min at 180 × *g*, and adjusted to a concentration of 1×10^5 to 2×10^5 cells per 100 μl of medium before use in a lymphocytotoxicity assay.

Lymphocytotoxicity assay. The details of this assay have been reported previously (12, 13). Results are expressed as a percentage of maximum ^{51}Cr released. $\%^{51}Cr$ released = 100 × {(radioactivity released by antiserum − radioactivity released in the absence of antiserum)/(radioactivity released by Nonidet-P40-solubilized cells − radioactivity released in the absence of antiserum)}.

RESULTS

Isolation of cross-reactive bacteria from $B27^+AS^+$ patients. In an initial study, it was shown that a cross-reactive *E. coli* in the bowel flora of a normal control patient ($B27^-AS^-$) was able to remove the lymphocytotoxic activity of a rabbit anti-*Klebsiella* sp. K43 BTS1 serum for $B27^+AS^+$ lymphoid cells in a ^{51}Cr release assay (12). We wondered whether the presence of cross-reactive bacteria was significantly higher in $B27^+$ individuals ($B27^+AS^-$) and in particular in $B27^+$ patients with AS ($B27^+AS^+$). Since *E. coli* was selected as representative of normal flora, it was necessary to demonstrate that random selection of bacterial colonies was sufficient to isolate cross-reactive bacteria from $B27^+AS^+$ patients and $B27^+AS^-$ controls. Rectal swabs from 10 $B27^+AS^+$ patients and 10 $B27^+AS^-$ controls were cultured on MacConkey agar plates. Five typical *E. coli* colonies were selected from each plate and grown overnight in Luria broth. Cultures were harvested, absorbed with cross-reactive antibacterial sera and then tested in the ^{51}Cr lymphocytotoxicity assay. All colonies (50 of 50) from $B27^+AS^+$ patients were able to absorb the lymphocytotoxic activity from these antisera, whereas no colonies (0 of 50) from the $B27^+AS^-$ controls removed this activity. This procedure demonstrated the validity of selecting one colony from an individual as representative of their bowel flora. Hence, only one colony per individual was selected for subsequent experiments.

In all patients (20 of 20) from whom rectal swabs were obtained, cross-reactive *E. coli* was present, whereas no cross-reactive organisms could be isolated from $B27^+AS^-$ individuals (Table 1). In light of these results, we examined a number of bowel flora from randomly selected patients for cross-reactive gram-positive organisms. Clostridial, streptococcal, and staphylococcal species were also able to absorb the lymphocytotoxic activity of anti-*S. typhimurium* BTS 69 sera. Antisera to previously characterized cross-reactive organisms were used interchangeably since they all specifically lyse $B27^+AS^+$ lymphocytes, that is to say, they are serologically indistinguishable (12) (Table 2). A list of the various cross-reactive bacterial species isolated from all patients is presented in Table 3.

Persistence of cross-reactive bacteria. Five patients were selected for a long-term follow-up for the presence of cross-reactive bacteria. In this ongoing investigation, the presence of randomly selected cross-reactive *E. coli* persisted for at least 9 months (Table 4). It should be noted that the $B27^-AS^-$ individual has maintained cross-reactive organisms for a period of 2 years. This individual, who is a member of our group, has worked with cross-reactive bacteria for the past 4 years. However, other members of our group (all $B27^-AS^-$) who have also been in contact with the cross-reactive bacteria did not appear to harbor such organisms on the four occasions over a 2-year period when rectal swabs were examined.

Lymphocytotoxic activity of anti-*Klebsiella* sp. K43 BTS1 sera for $B27^+AS^+$ cells. To assess the relevance of the presence in bowel flora of cross-reactive bacteria, we tested the lymphocytotoxic effect of cross-reactive antibacterial sera on the $B27^+AS^+$ cells of 20 patients from whom these bacteria were isolated. All cells of patients were lysed in the ^{51}Cr assay (Table 5). This effect was not seen with the cells of the $B27^-AS^-$ individual who harbors cross-reactive organisms nor with other members of our group who are HLA-$B27^-$.

The results presented above and in Tables 4 and 5 suggest a relationship between the presence of cross-reactive bacteria in AS patients and the presence on the cells of these individuals of a B27-associated determinant.

DISCUSSION

This study demonstrates, for the first time, the presence and maintenance of specific, cross-reactive, nonpathogenic bacteria in the bowel flora of patients with AS (Table 1). Further, we have concurrently shown that the cross-reactivity phenomenon is not confined to gram-negative enteric organisms but is continuously expressed by a range of gram-

TABLE 2. Absorption of the cytotoxic activity of anti-*S. typhimurium* BTS 69 sera by gram-positive bacteria isolated from rectal swabs of $B27^+AS^+$ patients

Bacteria	Target lymphocytes $B27^+AS^+$
None	65–85[a]
Klebsiella sp. K43 BTS1	0–5
Klebsiella sp. F77	75–87
Clostridia sp.	0–3
Streptococci sp.	1–6
Staphylococci sp.	0–4

[a] Percentage of maximum ^{51}Cr released measuring absorption of anti-*S. typhimurium* BTS 69. The range of percent lysis refers to results obtained with target cells through a series of assays. It was not possible to test all bacterial isolates at the same time.

TABLE 3. Cross-reactive bacterial[a] species isolated from rectal swabs of $B27^+AS^+$ patients

Gram-negative	Gram-positive
E. coli	*Streptococcus faecalis*
Klebsiella pneumoniae[b]	*Streptococcus faecium* var. *durans*
Proteus mirabilis[b]	*Streptococcus intermedius*
	Streptococcus mittus
	Staphylococcus epidermidis
	Clostridium perfringens

[a] The organisms listed in this table refer to the species designation of the bacterial genera presented in the text and Table 1. It should be noted that not all patients were screened for possible cross-reactive gram-positive bacteria.

[b] Although *Klebsiella* and *Proteus* species were not routinely tested, several colonies of these organisms (when isolated on MacConkey agar plates from the rectal swabs of $B27^+AS^+$ patients) were found to be cross-reactive with $B27^+AS^+$ cells. Hence, they have been included in this table.

positive organisms, including streptococcal, staphylococcal, and clostridial species (Table 2 and 3). The strong association of these nonpathogenic bacteria with AS patients suggests that the infectious process in this disorder may be different from other seronegative arthropathies, such as reactive arthritis, in that an acute episode of infection may not occur. The continued isolation of cross-reactive organisms over a period of 12 months (Table 4) would tend to argue for the possibility of a subtle, ongoing, "pathogenic" process.

It is significant that the lymphocytes of all patients from whom cross-reactive bacteria were isolated were lysed by the appropriate cross-reactive antisera in a ^{51}Cr lymphocytotoxicity assay (Table 5). However, the lymphocytes of a $B27^-AS^-$ individual were not susceptible to lysis despite the maintenance of these organisms for 2 years. In essence, these findings imply that there is a specific relationship between the presence of cross-reactive bacteria and the altered susceptibility of $B27^+$ host tissue. We have previously reported that $B27^+AS^-$ cells, when incubated in the presence of culture filtrates from cross-reactive bacteria, are rendered susceptible to lysis by anti-*Klebsiella* sp. K43 BTS1 sera (6, 7). In addition, the data presented here indicate that the B27-specificity is a necessary condition for the serologically detectable alteration of $B27^+$ cells. Nevertheless, any explanation for a pathogenic process in AS must account for the fact that the $B27^+$ cross-reactive determinant persists on the surface of $B27^+AS^+$ lymphocytes in vivo for many years after the acute onset of disease. It is interesting to note that four of the patients whose cells were tested in the lymphocytotoxicity assay have spent most of their lives in countries other than Australia. In view of the fact that we have recently been able to identify correctly, in a blind trial, a number of AS patients from the Dutch population (J. J. van Rood and A. van Leeuwen, personal communication), it would appear that this phenomenon is not restricted to our local patient population. Obviously, it would be most informative to study $B27^+$ clinically normal individuals from families with at least one family member with AS and who possess cross-reactive bacteria in their bowel flora. Their long-term follow-up may provide important epidemiological data which associates presentation of disease with the initial detection of these organisms. These studies are currently in progress.

In an attempt to provide an immunogenetic mechanism for the association of bacteria with $B27^+$ AS, we have postulated (14) that the in vivo persistence of the bacterial cross-reactive marker on $B27^+AS^+$ cells may be attributable to the acquisition of procaryotic DNA such as from a plasmid. Further, preliminary data indicate that an extrachromosomal element (plasmid) codes for this marker in *Klebsiella* sp. K43

TABLE 4. Persistence of cross-reactive bacteria in the bowel flora of five $B27^+AS^+$ patients and one $B27^-AS^-$ individual

Patient or control	Persistence at time after initial isolation (mo):		
	5	9	12
Patient[a]			
1	+[b]	NT[c]	+
2	+	NT	+
3	+	+	NT
4	+	+	NT
5	+	+	+
Control[d]			
A	+	+	+
B,C,D	−	−	−

[a] All patients were designated by numbers.

[b] Presence of cross-reactive bacteria was assessed by selecting *E. coli* colonies randomly from MacConkey agar plates, growing isolates overnight in Luria or nutrient broth and harvesting cultures for absorption with cross-negative antibacterial sera. Absorbed sera were then tested in a ^{51}Cr lymphocytotoxicity assay.

[c] NT, Not tested.

[d] HLA-$B27^-AS^-$ individuals have been designated by capital letters. The person, designated by the letter A, is the same $B27^-AS^-$ individual referred to in Table 1, footnote *c*. This person was also tested at 18 months and 24 months after initial isolation of cross-reactive bacteria. On all occasions, cross-reactive bacteria were present. Individuals designated B, C, and D were other members of our group (all $B27^-AS^-$).

TABLE 5. Lymphocytotoxicity of cross-reactive antibacterial sera for cells of $B27^+AS^+$ patients from whom cross-reactive *E. coli* was isolated

Targets lymphocytes from:	% Maximum ^{51}Cr released in presence of antisera raised against:	
	Klebsiella sp. K43BTS1	*Klebsiella* sp. F77[a]
Patient[b]		
1	75	5
2	68	4
3	83	12
4	92	12
5	91	4
6	86	9
7	78	10
8	80	14
9	79	6
10	79	6
11	80	12
12	85	19
13	72	19
14	73	6
15	82	5
16	76	12
17	59	0
18	74	6
19	75	10
20	70	16
Control[c]		
A	8	4
B	10	9
C	4	6
D	8	9

[a] *Klebsiella* sp. F77 is a non-cross-reactive isolate which does not cross-react with $B27^+AS^+$ cells.

[b] $B27^+AS^+$ patients were designated by numbers.

[c] $B27^-AS^-$ individuals who have worked with cross-reactive bacteria for at least 2 years.

BTS1 (J. S. Sullivan, J. K. Prendergast, and L. I. Upfold, unpublished data). The discovery that a range of unrelated enteric bacteria (e.g., *Salmonella* sp., *Shigella* sp., *Campylobacter* sp., and an enterotoxigenic *E. coli* strain) also shared the antigenic determinant suggests that the molecular basis of this association could be explained by the presence of a common plasmid. Alternatively, it is conceivable that the gene(s) coding for this surface molecule is carried by unrelated plasmids. Since it is well recognized that plasmids have a limited host range (3), the expression of the B27-associated determinant by gram-positive bacteria is, at first, puzzling. It would, therefore, seem improbable that a common plasmid found in such a wide range of human autochthonous bacteria (Table 2) is responsible for the production of this determinant. However, the broad-spectrum transference of this genetic trait may be associated with a mobile, self-replicating, genetic element such as a transposon. The host range of transposons is recognized to be broader than that of any plasmid with which they are commonly associated (4).

The availability of a pool of exogenous DNA from nonpathogenic cross-reactive bacteria, which may be present in the bowel flora of $B27^+$ individuals without evidence of disease, offers an interesting pathogenetic mechanism(s) by which the uptake and expression of procaryotic DNA may alter the surface of $B27^+$ cells (11; L. I. Upfold, J. S. Sullivan, J. K. Prendergast, and A. F. Geczy, Prog. Allergy, in press). This cell-surface modification may be an important early event in the pathogenesis of this disease.

ACKNOWLEDGMENTS

This work was supported in part by grants from the National Health and Medical Research Council of Australia and from the Phillip J. Benjamin Memorial Research Grant, New South Wales, Australia.

We thank George Struthers, Department of Rheumatology, The Medical School, University of Birmingham, Birmingham, United Kingdom, for the isolation of clostridia, and Gabriel Morris and Michael Toohey, Institute of Clinical Pathology and Medical Research, Westmead Centre, Parramatta Hospital, Sydney, Australia, for positive confirmation of gram-positive bacterial isolates. We express our appreciation to Leanne Wood for excellent secretarial assistance.

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