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HLA-B27 associated cross-reactive marker on the cells of New Zealand patients with ankylosing spondylitis

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SUMMARY We have previously shown that antibodies raised in rabbits to certain enteric bacteria will specifically lyse, in a ^{51}Cr release assay, the peripheral blood lymphocytes (PBL) of 80% of HLA-B27 positive patients with ankylosing spondylitis ($B27^+AS^+$) but not the PBL of HLA-B27 positive normal controls ($B27^+AS^-$). Other laboratories have been unable to reproduce these findings. This study was designed to ascertain whether this lack of reproducibility was due to a peculiarity of our $B27^+AS^+$ patients or to technical difficulties in the complement mediated ^{51}Cr release assay. We have shown in this blind study that the PBL of 16 out of 18 $B27^+AS^+$ patients from a New Zealand population were lysed by our antisera but none of the PBL of 20 $B27^+AS^-$ normal controls were lysed. The phenomenon of 'cross reactivity' between certain enteric bacteria and $B27^+AS^+$ PBL is not confined to the Sydney AS population.

Key words: enteric bacteria, lymphocytotoxicity, seronegative arthritis.

Although interest in the association between HLA-B27 and ankylosing spondylitis (AS) has been intense, the mechanism by which the HLA-B27 gene or its product predisposes to the development of the disease remains unknown.

The initial observations of Ebringer *et al.*[1] on the possible relationship between enteric organisms and AS prompted our group to search for 'cross reactivity' between Gram-negative bacteria and cells from patients with AS. We have been able to show that antisera raised in rabbits, initially to certain strains of klebsiella and subsequently to a number of enteric organisms, are able to recognise and lyse the peripheral blood lymphocytes (PBL) of 70–80% of patients with HLA-B27 positive ankylosing spondylitis ($B27^+AS^+$) but not those of HLA-B27 negative spondylitics ($B27^-AS^+$) or of the cells of HLA-B27 positive or negative controls ($B27^+AS^-$ and $B27^-AS^-$ respectively).[2] Despite considerable early interest, these findings came to be regarded with some scepticism because other laboratories were unable to raise antisera which reproduced our findings in a lymphocytotoxicity assay.[3 4]

The aim of the present study was firstly to attempt to validate our initial observations in an entirely new group of patients, and secondly, by using this different population group, to determine whether the failure of others to reproduce our findings was due either to geographic variations in the expression of the B27 associated cross-reactive marker or to technical aspects of the lymphocytotoxicity assay. The study was structured to ensure complete objectivity in the correlation of clinical status and assay results by the use of a random code, held by one of the group, and broken only after all the laboratory and clinical data had been collected.

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Patients and methods

PATIENTS

Patients with AS were selected from the rheumatology outpatients attending the Auckland or Middlemore hospitals in Auckland, New Zealand. The normal controls were selected from healthy HLA-B27 positive blood donors in the same city. The

Auckland population was chosen for several reasons: (*a*) cooperation of the rheumatologists and blood transfusion centre allowed ready availability of patients and controls; (*b*) this population represented a discrete cohort of individuals quite different from the Sydney population on which our previous work was based; and (*c*) twice-daily air services between Auckland and Sydney provided rapid transfer of blood to the Sydney Red Cross Blood Transfusion Service.

From a group of 60 AS patients previously assessed clinically and radiologically[5] 20 patients were chosen on the basis of their availability and the unequivocal nature of the diagnosis. Two $B27^-AS^+$ patients were included in the group. All 20 AS patients satisfied New York criteria.[6]

Twenty-five $B27^+AS^-$ individuals identified by the Blood Transfusion Centre in Auckland were contacted and asked to participate in the study as controls; 20 presented for assessment.

A relevant medical history, including the intake of non-steroidal anti-inflammatory drugs (NSAID), was obtained and the range of back and neck movements was assessed by standardised techniques in both patients and controls.[7] These measurements were then compared with standards of age and sex matched controls.[7] No radiographs were performed on the normal individuals. The characteristics of the patients with AS and of the $B27^+AS^-$ control group are shown in Table 1.

Table 1 *Characteristics of AS patients and HLA-B27 positive controls*

	AS patients	*Controls*
Number	20	20
B27+	18	20
Sex		
Male	14	18
Female	6	2
Age (mean)	47·4	36·9
Range	30–63	23–64
Disease (mean duration)	25·8 years	—
Range	6–40	
Number with peripheral arthritis at time of study	0	0
Number with restricted range of neck movement	16	0
Number with restricted range of back movement in three planes	14	0
Number with chest expansion <2·5 cm	9	0
Number with chronic thoracolumbar back pain	20	2
Number taking NSAID within one month of study	15	1

METHODS

From each patient and control individual 50 ml of venous blood was obtained and placed in sodium heparin tissue typing tubes. The blood was transferred by air in an insulated container to Sydney where, on arrival at the Red Cross Blood Transfusion Service, it was randomly coded. The code was held by Dr H Bashir and was not broken until all cytotoxicity assays and clinical evaluation had been completed.

Cell preparation

The PBL were separated from the blood by centrifugation in Ficoll-Paque (Pharmacia) for 25 min at 580 *g*. The leucocyte band was removed and washed with RPMI 1640 (Flow) medium containing 2 ml heparin (1000 units/ml), 10 ml 1 M HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid, Sigma) buffer, 5 ml L-glutamine (200 mM), and 2 ml penicillin-streptomycin (5000 IU, 5000 μg/ml; Flow) per 500 ml of RPMI medium. The leucocytes were suspended in RPMI 1640 medium containing 10% human group A serum and then centrifuged for a further 10 min at 180 *g*. The leucocytes were resuspended in RPMI 1640 medium containing 10% group A serum and adjusted to a cell concentration of 10^7/ml of medium. The cells were placed in an ice bath and an equal volume of 20% dimethyl sulphoxide in RPMI was added. All reagents and pipettes were cooled to 4°C before this addition. The cells were then placed in 2 ml freezing vials in aliquots of 1 ml each containing 5×10^6 cells. These vials were immediately placed in a controlled freezing cycle, frozen to −100°C, and then stored in liquid nitrogen.

Antiserum production

An *Escherichia coli,* previously identified as being capable of producing lymphocytotoxic rabbit antisera specific for $HLA\text{-}B27^+AS^+$ lymphocytes, was used to produce antisera.[8] Other isolates of *E. coli* shown not to have this ability were used to raise negative antisera.[8] These organisms were killed by adding 0·25 ml of 40% formaldehyde to a 20 ml suspension of the organisms (10^9–10^{10}/ml) in nutrient broth. The bacteria (10^9–10^{10}) killed by formalin were washed twice with sterile 0·9% NaCl and mixed with 2 ml of complete Freund's adjuvant (Difco). This mixture was injected subcutaneously and intramuscularly into multiple (i.e., more than four) sites on rabbits (3–5 kg), whose serum had previously been shown not to contain antilymphocytic activity. Two weeks later each rabbit was boosted with a mixture of organisms and complete Freund's adjuvant, and 10–12 days later the animals received a further inoculation of cells in complete Freund's

adjuvant. Four weeks after the initial injection the rabbits were bled and the serum stored at −30°C until used in the cytotoxicity assay.

Complement mediated lymphocytotoxicity assay

Positive and negative antisera, as defined above, were tested on the PBL of the $B27^+AS^+$ and $B27^-AS^+$ patients and $B27^+AS^-$ controls. The frozen PBL were thawed in a water bath (40–44°C) and gently agitated until the cell pellet was almost completely thawed. Each pellet was transferred to a 30 ml plastic tube where 1 ml of human group A serum was added dropwise followed by 1 ml of RPMI 1640 medium. The tube was immediately filled with RPMI 1640 medium and spun at 180 *g* for 10 min. The cells were then resuspended in 10 ml of RPMI 1640 medium containing 10% group A serum and washed to remove any further trace of dimethyl sulphoxide. After this final wash the cells were resuspended in 1 ml of RPMI 1640 containing 10% human group A serum and 100 μCi of ^{51}Cr (specific activity: 200–500 μCi/μg; Amersham) for 60 min at 37°C. The cells were then washed twice with RPMI 1640 medium and resuspended in RPMI 1640 containing 10% human group A serum at a concentration of 2×10^6 cells/ml. 100 μl of this ^{51}Cr-labelled cell suspension was added to 10 × 75 mm conical plastic tubes. The cells were incubated with 100 μl of antiserum (undiluted) at 20–26°C for 40 min. Rabbit complement (100 μl; Pel-Freez Biologicals) was added to each tube and the incubation continued for a further 75 min. After the incubation with complement the cells were centrifuged at 400 *g* for 10 min and 100 μl of the supernatant counted in a Beckman auto-gamma scintillation spectrometer. The amount of radioactivity in the samples was compared with the total release of ^{51}Cr from PBL (100 μl) in the presence of 200 μl of 1% Nonidet P–40 (NP40), while background release (i.e., release in the absence of antiserum) was calculated from the ^{51}Cr released from the PBL (100 μl) in the presence of complement (100 μl) and RPMI 1640 containing 10% human group A serum (100 μl).

The results were expressed as the percentage of maximum ^{51}Cr released, which was calculated as follows:

$$\%\ ^{51}Cr\ \text{release} = 100 \times \frac{\text{Radioactivity released by antiserum} - \text{Radioactivity released in absence of antiserum}}{\text{Radioactivity released by NP40 solubilised cells} - \text{Radioactivity released in absence of antiserum}}$$

Positive and negative antisera were used to test each patient's cells. All tests were done in duplicate and the cytotoxicity averaged. We have previously stated that ^{51}Cr release values > 50% are considered positive and values <20% are negative.[8] All lymphocytotoxicity assays were performed by one of us (LMcG).

Assays were done once only on the cells of each patient and control, with the exception of one assay involving the cells of eight patients that had to be repeated because of unacceptably high background readings (i.e., >80% of the NP40 total release).

Results

The results of the ^{51}Cr release cytotoxicity assays are diagrammatically presented in Fig. 1. It can be seen that the PBL of 16 of the 18 $B27^+AS^+$ patients showed cytotoxicity values ≥48·6%. The remaining two $B27^+AS^+$ and both the $B27^-AS^+$ patients had values of <20%.

There were no $B27^+AS^-$ controls whose cells gave cytotoxicity values >20% in the presence of positive antisera. The cytotoxicity values produced by the negative antiserum were <23% in all 40 individuals tested (data not shown).

The two $B27^+AS^+$ patients whose PBL showed <20% ^{51}Cr release could not be distinguished clinically from the other patients. Both were taking regular NSAID medication. It is pertinent that of the five AS patients not taking NSAID medication four showed values of >50% ^{51}Cr release, while the other patient who was HLA-B27 negative had <20% ^{51}Cr release.

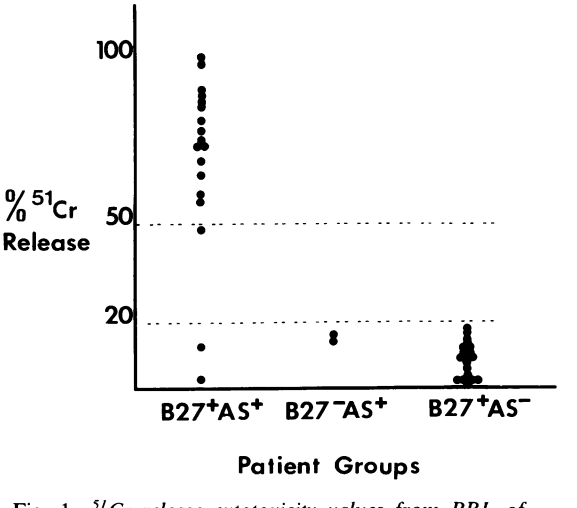

Fig. 1 *^{51}Cr release cytotoxicity values from PBL of $B27^+AS^+$, $B27^-AS^+$ patients and $B27^+AS^-$ normal controls.*

Discussion

It was the aim of this study to determine whether the inability of other groups to reproduce our results[3 4 9] was due either to an HLA-B27 associated antigenic determinant peculiar to the Sydney AS population or to technical problems associated with the cytotoxicity assay. The results described for the groups of $B27^+AS^+$ and $B27^-AS^+$ patients and the $B27^+AS^-$ controls allow several conclusions.

We have been able to confirm in an entirely new population our initial observations that certain antibacterial sera recognised and lysed PBL from more than 80% of $B27^+AS^+$ individuals but not the cells from $B27^-AS^+$ patients or $B27^+AS^-$ as controls.

To eliminate any possibility that the patients' clinical status might influence the interpretation of the assay result the assays were performed in a 'blind' manner. This was done by randomly coding blood samples as they arrived from New Zealand and having one member of the group (HVB), who was uninvolved in either the clinical assessment or the cytotoxicity assays, break the code only after all clinical and laboratory data had been finalised. It is clear from this study and from the work of Archer *et al.*,[10] reporting similar findings in a group of London AS patients, that the 'cross reactivity' phenomenon is not restricted to AS patients in Sydney.

It has previously been suggested that age and medication disparities between our $B27^+AS^+$ patients and $B27^+AS^-$ controls could be responsible for our results.[11] Although it is true that the $B27^+AS^+$ group in this study is older on average than the $B27^+AS^-$ group, their age range is similar, and it would be highly contentious to suggest this age disparity could be responsible for such a marked difference in the lymphocytotoxicity values we have reported. Similarly, it is very unlikely that NSAID treatment could be responsible for this phenomenon as there were four $B27^+AS^+$ patients who were not taking these medications and whose PBL gave high levels of lymphocytotoxicity and one $B27^+AS^-$ normal donor who was taking NSAID medication whose PBL gave low levels of lymphocytotoxicity.

Thus it is possible to conclude that the failure of certain groups to reproduce our findings is unlikely to relate either to racial or geographic variations in the patients' expression of the HLA-B27 associated cross-reactive determinant or to drug or age related effects. It seems more likely that the difficulty relates to problems in raising antiserum or to the technique of the cytotoxicity assay itself. In that regard it should be noted that one of the nine assays performed in this study failed, in that very high background readings gave uninterpretable results. Although we have not quantified in detail the relative importance of each variable in this assay system, this failure highlights the fact that all components, including the condition of the PBL, the media, and the complement, are critical to the outcome of the assay.

The explanation for the lack of corroboration by others is not clear. Perhaps it could be that there is a low density of the HLA-B27 associated cross-reactive marker on $B27^+AS^+$ patients' cells, which may occasionally escape detection by some antisera. To minimise this possibility we always use undiluted sera in this assay. It could be argued that we may have been fortuitous in raising these specific antisera by our immunisation protocol, but as we have been able to raise antisera to a number of cross-reactive enteric organisms, all with the same specificity in over 22 different rabbits and several guinea pigs, it should be possible to repeat the procedure in other laboratories. However, until other groups are able to raise specific antisera to these organisms this relationship between AS and bacteria will remain controversial.

Even if the lymphocytotoxicity assay system can be reproduced by others it is not certain whether this relationship between enteric bacteria and AS is of pathogenetic significance to the disease process. Although we have previously presented some evidence as to how the cross-reactive determinant may be passed from bacteria to patients' cells,[12] we do not know whether the presence of this determinant brings about the development of the pathological lesions of AS. We hope the possible mechanisms whereby this could occur will be investigated in detail when the relationship between these cross-reactive bacteria and AS gains wider acceptance.

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