

# B-cell antigen D8/17 is a marker of rheumatic fever susceptibility in Aboriginal Australians and can be tested in remote settings

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It has long been observed that rheumatic fever (RF) appears to occur only in susceptible individuals. This is based on observations of a familial association,<sup>1</sup> controlled studies<sup>2,3</sup> and the fact that only a limited proportion of individuals exposed to rheumatogenic Group A streptococci subsequently develop RF.<sup>4,5</sup>

Various potential markers of susceptibility to RF have been investigated, including blood groups and human leukocyte antigen (HLA) subtypes, but findings have often been inconsistent.<sup>6-8</sup> A non-HLA B-cell antigen known as D8/17 has been investigated in several population groups. A study of American and Caribbean people showed that, in healthy individuals, a mean of 6.12% of B cells were positive for D8/17, compared with 33.5% in patients with a past history of RF ( $P < 0.0001$ ). Intermediate levels were seen in unaffected first-degree relatives of index cases, suggesting that D8/17 might be an inherited factor.<sup>9</sup> Subsequently, D8/17 was found to be highly sensitive as a marker for previous RF in the United States, Mexico, Russia, Chile, and Israel.<sup>10,11</sup> However, in north India it was less sensitive than other, locally derived B-cell markers.<sup>12-14</sup>

The World Health Organization recognises an urgent need to develop new strategies to control RF.<sup>15</sup> The ability to identify a subgroup of individuals at increased risk of RF could help direct primary prevention programs.<sup>15</sup>

The D8/17 non-HLA B-cell antigen has not been studied in Australia, where Aboriginal people have among the world's highest prevalence of rheumatic heart disease (RHD).<sup>16</sup> Populations with the highest rates of RF and RHD often live far from laboratories with the technology to perform D8/17 assays. Assays for D8/17 expression described to date usually require testing of fresh whole blood within 1 or 2 hours of sample collection, which has made it difficult to assess D8/17 expression in people living in remote settings. We aimed to study the expression of the B-cell marker D8/17 in Australians with a past history of RF or RHD, their relatives and healthy people. We also studied some technical modifications to the D8/17 assay that

## ABSTRACT

**Objective:** To test the B-cell antigen D8/17 as a marker of past rheumatic fever (RF) in a predominantly Aboriginal Australian population, and to evaluate technical modifications to allow its use in remote settings.

**Design and setting:** Cross-sectional survey in a remote Aboriginal community, a regional tertiary referral hospital and a tertiary paediatric centre in Melbourne.

**Participants:** 106 people, including three with acute RF, 38 with a history of past RF, 20 relatives of these people, and 45 healthy controls.

**Main outcome measure:** D8/17 expression in B cells.

**Results:** Blood was collected from each participant and the expression of D8/17 and CD19 in each sample was analysed by flow cytometry. The mean proportion of D8/17-positive B cells was 39.3% (SD, 11.8) in patients with previous RF, 22.5% (SD, 5.2) in first-degree relatives, 11.6% (SD, 7.2) in controls, and 83.7% (SD, 10.1) in patients with acute RF (analysis of variance test between means,  $P = 0.001$ ). A cut-off of 22.1% of D8/17-positive B cells to indicate past RF yielded the highest percentage of correct results (95.4%). Delayed staining of whole blood (mean, 0.55 days; SD, 0.2) gave equivalent results to immediate staining, but the D8/17 assay on peripheral blood mononuclear cells was unreliable.

**Conclusions:** The B-cell antigen D8/17 accurately identifies Australians with a past history of RF, and the assay is feasible in remote settings with access to facilities capable of performing D8/17 staining within half a day of sample collection.

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might allow it to be used for people living in remote settings.

## METHODS

Participants were enrolled in three centres:

- a remote Aboriginal community in the Northern Territory;
- a regional tertiary referral hospital; and
- a tertiary paediatric centre in Melbourne.

Patients with acute RF, past RF (with or without RHD), healthy relatives of these patients and healthy volunteers unrelated to the patients were recruited. The diagnosis of previous RF was confirmed from medical notes. In most cases the Jones criteria<sup>17</sup> were supplemented with an echocardiogram report consistent with RHD. The absence of a history of RF or RHD in relatives and controls was documented prospectively by interview with the individual, review of medical notes and cardiac auscultation. Relationship status was determined by a detailed discussion in the local language.

Ethical approval was obtained from the Human Research Ethics Committee of Territory Health Services and Menzies School of Health Research. Participants were educated

about the study in the local language and written consent was obtained.

## D8/17 staining and flow cytometry

Blood was collected in lithium heparin vacutainers (Becton Dickinson Australia, Sydney, NSW). Patients' diagnostic categories were concealed from laboratory staff. The flow cytometry assay for D8/17 as described by Chapman et al was used.<sup>18</sup> In brief, either 20  $\mu$ L of mouse anti-D8/17 monoclonal antibody (provided by Professor J Zabriskie, Rockefeller University, New York, USA) or 5  $\mu$ L of mouse IgM, lambda (isotype control; Sigma, Sigma-Aldrich, Sydney, NSW) was added to 300  $\mu$ L of whole blood. After 40 minutes' incubation at 4°C, the samples were washed with phosphate buffered saline (PBS). Fluorescein-5-isothiocyanate (FITC)-labelled goat anti-mouse IgM ( $\mu$ ) antibody (Caltag, Burlingame, Calif, USA) and phycoerythrin-labelled anti-CD19 (Becton Dickinson Australia, Sydney, NSW) were added. After 20 minutes' incubation at room temperature, red cells were lysed with ammonium chloride lysis solution (150 mM ammonium chloride, 10 mM potassium

**1 Demographic features of participants**

Demographic feature	Category					Total
	RF	RHD or past RF	First-degree relative	Second-degree relative	Control	
Ethnicity						
Aboriginal	3	34	7	8	35	87
Non-Aboriginal	0	4	5	0	10	19
Sex						
Male	1	16	3	0	22	42
Female	2	22	9	8	23	64
Mean age in years (range)	10 (8–14)	36 (19–52)	37 (11–58)	34 (19–68)	39 (4–63)	—
<b>Total</b>	<b>3</b>	<b>38</b>	<b>12</b>	<b>8</b>	<b>45</b>	<b>106</b>

RF = rheumatic fever. RHD = rheumatic heart disease. ◆

bicarbonate, 1 mM tetrasodium EDTA) for 15 minutes. Cells were centrifuged and washed with PBS before resuspension in PBS for analysis by flow cytometry.

All analysis was done on a FACS Calibur flow cytometer (Becton Dickinson Australia, Sydney, NSW). Lymphocytes were gated according to their forward and side scatter profile. To eliminate background fluorescence, values were set for the quadrants by defining the isotype control samples (no D8/17 antibody) as 99% negative. The fluorescence was measured and the percentage of D8/17-positive cells of a set number of CD19-positive cells was calculated with FlowJo software (Tree Star, San Carlos, Calif, USA).

**Preparation of peripheral blood mononuclear cells**

For a subset of samples, peripheral blood mononuclear cells (PBMCs) were isolated on a Ficoll-Paque PLUS density gradient (Amersham Pharmacia Biotech, Uppsala, Sweden), resuspended in RPMI (Roswell Park Memorial Institute) medium (Gibco, Invitrogen Australia, Melbourne, VIC), 50% fetal calf serum, 10% dimethylsulfoxide (Sigma, Sigma-Aldrich, Sydney, NSW), and slowly frozen to -80°C. Frozen PBMCs were thawed, incubated with antibodies, and analysed by flow cytometry as described above. Results obtained using PBMCs were compared with the results obtained by analysis of fresh whole blood from the same patient.

**Assessing the effect of delayed staining**

Transportation of specimens from remote locations involved delays of up to a day as well as temperatures in excess of 30°C on

occasion. To assess any effect of time delay artefact on the accuracy of the D8/17 assay, 38 samples were stained on location in the community and compared with results obtained by staining the samples in the laboratory after a delay of about half a day.

**Statistical analysis**

Based on published values for D8/17,<sup>9</sup> a sample size of only two patients per group was needed to differentiate mean B-cell D8/17 expression between patients with RF or RHD and control patients, and only four patients per group were needed to differentiate between patients with RF or RHD and unaffected family members (power, 90%; two-sided  $\alpha$ , 0.05).

The results were analysed using the Stata 7 statistical package (Stata Corporation, College Station, Tex, USA). The mean and standard deviation of the percentage of the B cells positive for D8/17 were calculated for the samples from patients in each category. Analysis of variance (ANOVA) was performed to determine whether a significant difference existed. The differences between the means of each of the categories and 95% confidence intervals were calculated. The relationship between the results obtained from first-degree and second-degree relatives were compared by means of Student's *t* test. A receiver operator characteristics (ROC) curve was constructed from the results from patients with RF or RHD and control patients. Results from immediate and delayed staining were compared graphically, including a Bland-Altman plot.

**RESULTS**

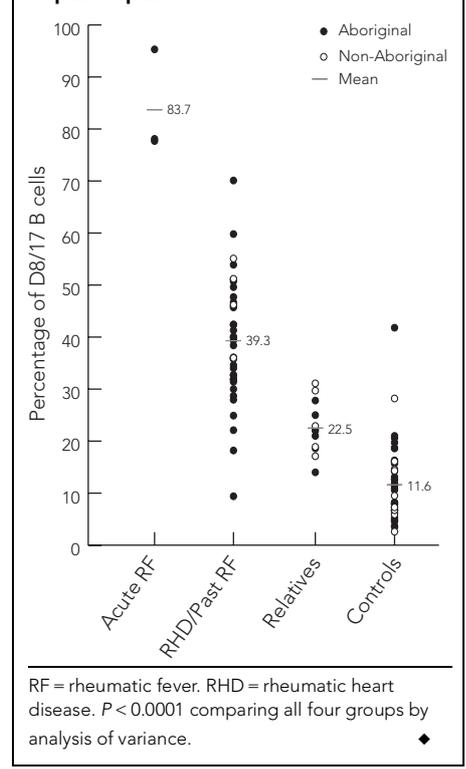
One hundred and six individuals were enrolled; all but 19 were Aboriginal (Box 1).

Participants included 42 males and 64 females; the proportions of males and females did not differ significantly between the groups.

Box 2 and Box 3 show that D8/17 expression was different in the four groups (ANOVA,  $P < 0.0001$ ) — patients with acute RF had the highest levels (mean, 83.7%; SD, 10.1), followed in descending order by patients with RHD or past RF (mean, 39.3%; SD, 11.8), relatives (mean, 22.5%; SD, 5.2) and controls (mean, 11.6%; SD, 7.3). Aboriginal or non-Aboriginal ethnicity appeared to have no effect on D8/17 expression. The difference in mean D8/17 level between first-degree relatives and second-degree relatives (22.5% [SD, 5.2] v 16.8% [SD, 8.7]) approached significance (*t* test,  $P = 0.08$ ), so only first-degree relatives were included in the analysis. The differences of the means of all groups were significant and substantial (greater than 10%).

In the ROC analysis, patients with either acute RF or a history of past RF or RHD were defined as positive cases and were compared with healthy controls. Based on this analysis, a cut-off of greater than 22.1% of B cells positive for D8/17 yielded the greatest percentage of accurate results (95.4%), with a sensitivity of 95.1% and a

**2 Percentage of D8/17-positive B cells in the four groups of participants**



**3 Comparison of mean D8/17 expression between categories of participants**

Categories compared	Difference of the mean percentage of D8/17-positive B cells (95% CI)
Acute RF and controls	72.0 (63.1–81.0)
Acute RF and RHD/ past RF	44.4 (30.2–58.7)
RHD/past RF and controls	27.6 (23.4–31.8)
RHD/past RF and relatives	16.7 (9.6–23.8)
Relatives and controls	10.9 (6.4–15.4)

RF = rheumatic fever. RHD = rheumatic heart disease. ◆

specificity of 95.6%. The likelihood ratio of a positive test at this cut-off was 21.4. Thus, a patient's likelihood of having the disease with a D8/17-positive B-cell level greater than 22.1% was 21.4 times the pretest probability. If a negative result (D8/17-positive B-cell level less than 22.1%) is obtained, the pretest probability is multiplied by 0.05.

Box 4A shows a good correlation between the results for the D8/17 analysis of blood stained immediately and that stained with a delay of about half a day (mean, 0.55 days; SD, 0.2) for 38 sample pairs (correlation coefficient [r] = 0.94). A Bland–Altman plot (Box 4B) confirmed that differences between the two methods were not related to the level of D8/17 positivity. There was poor correlation between the results of the D8/17 assay obtained from analysis of fresh whole blood compared with frozen PBMC, in 19 sample pairs (r = 0.32).

**DISCUSSION**

Our findings indicate that the B-cell antigen D8/17 is a sensitive and specific marker of past RF in Australians and are consistent with those of researchers in the United States, Mexico, Russia, Chile and Israel.<sup>9-11,19,20</sup> This adds to the growing evidence that D8/17 may be a universal marker of past RF in ethnically disparate populations. Negative findings by some US and north Indian researchers remain unexplained, but may reflect ethnic differences in the expression of B-cell antigens, selection of control patients, variation in antibody quality, or technical issues relating to the technique for selecting B cells.<sup>12-14,21</sup>

In previous reports, first-degree relatives have been shown to express D8/17 at intermediate levels, averaging 14.0% of B cells.<sup>9</sup> Intermediate expression of D8/17 was also demonstrated in the relatives of patients with Sydenham's chorea.<sup>20</sup> These findings suggest that the B-cell marker is inherited, possibly along autosomal recessive lines. In our study, the absolute percentages of B cells positive for D8/17 in each category (patients with a past history of RF or RHD, first-degree relatives and healthy controls) reflect previous results and increase their reliability and generalisability.<sup>9</sup>

It is clear that D8/17 expression is not merely a marker of streptococcal infection, as it is not increased in poststreptococcal glomerulonephritis and uncomplicated streptococcal tonsillitis.<sup>9,22</sup> However, post-streptococcal reactive arthritis is associated with elevated levels of D8/17, with D8/17 expression not conclusively differentiating between RF and poststreptococcal reactive arthritis in one small series.<sup>23</sup>

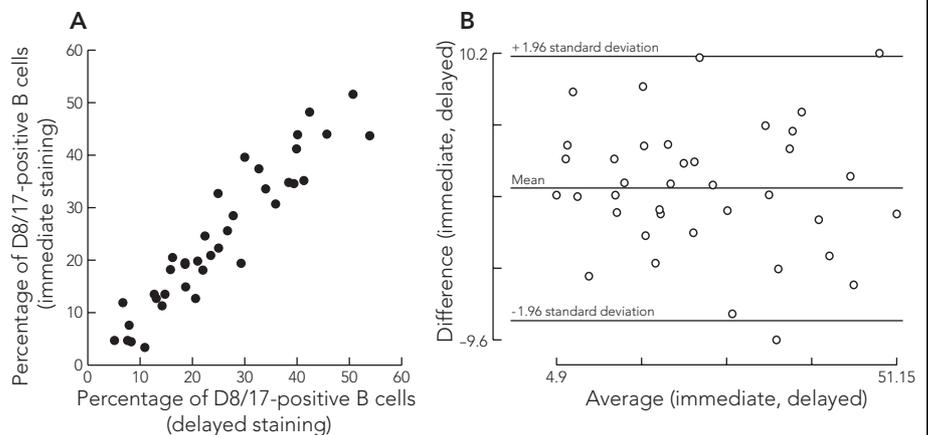
The functional significance of the D8/17 protein remains unknown. It does not match any known protein or HLA.<sup>20,24</sup> The antibody to D8/17 has been shown to cross-react with cardiac muscle, skeletal muscle, smooth muscle cells of blood vessels and recombinant M6 streptococcal protein. Adsorption testing showed that B cells from a patient with RHD inhibited the binding of the D8/17 antibody to cardiac muscle. It was thus suggested that the D8/17 antigen may act as a streptococcal binding site on the B

cells, and consequently become up-regulated after an infection, with B cells acting as antigen-presenting cells and influencing T-cell-specific cytotoxicity to heart and brain cells.<sup>22</sup>

For the first time, we evaluated D8/17 as a diagnostic test for RF or RHD by looking at its receiver operator characteristics, and found that a cut-off of 22.1% cells positive for D8/17 was the most accurate break point to distinguish between affected patients and healthy controls. Clearly, a positive test result is not diagnostic of RF or RHD, as 50% of unaffected first-degree relatives and 4% of healthy controls were found to be positive for D8/17. Rather, D8/17 may be seen as a marker of susceptibility to RF. The intermediate level of expression of D8/17 in unaffected first-degree relatives supports the conjecture that susceptibility is inherited rather than acquired. The consistency of the degree of elevation of D8/17 in first-degree relatives, but not controls, in a region where Group A streptococcal (GAS) infection is endemic suggests that inheritance, rather than environment or exposure to GAS, is the cause of the observed difference. Conclusive proof requires a prospective study of D8/17 expression in a high-risk population.

If D8/17 is an inherited marker of susceptibility to RF, it has a number of potential useful clinical applications. A cut-off point could be selected that could "rule out" past or current RF, which would be particularly useful in remote-living patients. In populations with a high incidence of RF, D8/17

**4 Comparison of immediate versus delayed staining**



**A:** Comparison of D8/17 analyses with immediate versus delayed staining.

**B:** Bland–Altman plot of immediate and delayed staining results. This plot is used to look for systematic differences when comparing two measurement techniques and to determine if the difference in results between the two techniques may be clinically important. In this case, the results with immediate and delayed staining were within 18% of each other on 95% of occasions. ◆

could be used as a screening test to identify a group at increased risk of developing RF, who may be suitable for a primary prevention intervention, such as any future GAS vaccine.

A hypothesis is that D8/17, already expressed in excess in susceptible patients, is further augmented by the process that leads to RF. This suggests that D8/17 might be incorporated into the Jones criteria for the diagnosis of acute RF,<sup>17</sup> either as an additional major manifestation, or as an obligatory feature in all diagnoses of acute RF. We are presently evaluating this in a larger, prospective study.

For clinical applicability, a diagnostic test must be readily available and cost effective. We looked at one way of reducing the overheads of this technically sophisticated test — storing specimens as frozen PBMC so that they could be tested in batches. We found that flow cytometry for D8/17 was not reliable when performed on frozen PBMC stored at  $-80^{\circ}\text{C}$ . However, we showed that a delay of up to half a day, such as that required to transport whole blood from a remote community to a central laboratory, had no significant effect on the accuracy of D8/17 analysis. As flow cytometry is not available in many locations where RF is common, further studies should investigate quantitative (rather than simple positive or negative) D8/17 assays that are cheap and practical in remote settings.

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## COMPETING INTERESTS

None identified.

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