Type-specific antibodies to purified streptococcal M proteins from potentially rheumatogenic M-types in patients with rheumatic fever and rheumatic heart disease

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This study was designed to identify the predominant serotypes of group A streptococci (GAS) responsible for rheumatic fever (RF) and rheumatic heart disease (RHD) in India. RF and RHD sera were screened for antibodies to M proteins retrospectively against known rheumatogenic M types and M types isolated from patients with acute RF. All GAS strains isolated from four patients with acute RF in a short outbreak of RF, were identical – serum opacity factor (SOF) negative, T-pattern 3/13 B3264 and M-non-typable. Because of this, M protein was isolated from only one of these four M-non-typable strain (S-399). This was done by limited pepsin digestion and purification by ion-exchange chromatography on DEAE-Sephadex, followed by gel filtration. Purified M protein was found to be homologous on SDS-PAGE (mol.wt 20 kDa), immunogenic in rabbits and to retain its antigenic structure. This purified M protein from an M-non-typable strain (pep-MNT) was used as an antigen to screen RF and RHD sera retrospectively by ELISA for the prevalence of the M-non-typable GAS strain. The prevalence of the M-non-typable strain was compared with that of the known rheumatogenic M types. Results suggest that the M-non-typable strain could be a provisional new rheumatogenic M type in India and could be a candidate for a multivalent M-protein vaccine to control RF and RHD.

Introduction

Acute rheumatic fever (RF) is a delayed, nonsuppurative sequel of upper respiratory infection with group A streptococci (GAS). RF and rheumatic heart disease (RHD) continue to be major health problems in children around the world and are widely prevalent in India, where the incidence is reported to be 6–11% among 5–15-year-old schoolchildren [1]. The risk of an acute RF attack following upper respiratory tract infection is 3–5% in endemic conditions [2, 3]. In developing nations, efforts to control RF and RHD are made by mass prophylaxis with appropriate antibiotics to eradicate initial streptococcal infections, but such programmes have met with only limited success [4]. Therefore, there is a need to develop a safe and effective multivalent vaccine against GAS to provide long-lasting immunity against those strains that may give rise to RF and RHD. The principal virulence factor of these organisms is known to be M protein and type-specific antibodies against this cell-wall antigen confer protective immunity. The formulation of an effective polyclonal vaccine requires knowledge of prevalent rheumatogenic M serotypes in any geographical area. Because the isolation of streptococcal strains from throat cultures from RF patients is only 2–3%, [5] an alternative approach was used in an attempt to identify rheumatogenic M types in India. This was done by identifying by ELISA type-specific M antibodies against known M serotypes as well as those isolated from acute RF cases in sera of RF and RHD patients. The ELISA-positive sera were verified by the opsonophagocytic test for type-specific antibodies.
Materials and methods

Patients and controls

Two-hundred clinically diagnosed RF cases and 200 RHD cases from Kalawatisaran Children's Hospital, G.B. Pant and RML Hospitals All India Heart Foundation, constituted the study material. Seventy-five control subjects were selected from school children aged 5–15 years in whom no streptococcal infection was observed either bacteriologically or serologically.

Culture

Throat swabs were taken from patients and controls. GAS isolates were studied for their T pattern, SOF production and M type, by standard procedures [6, 7].

Blood collection

Blood samples were collected from each patient in parallel with throat culture, followed by a second sample after 21–28 days.

M-non-typable strain

M protein was purified from a GAS strain that was SOF negative, T-pattern 3/12/B3264 and M-non-typable. This strain was isolated for four patients with acute RF in a short outbreak. The cells were grown in 20 L batches of Todd-Hewitt Broth (Difco) supplemented with yeast extract 0.2%. Cells were harvested by centrifugation and washed twice with 20 mM PBS, pH 7.4, and once with 67 mM phosphate buffer (PB), pH 5.8.

Extraction of crude M protein

M protein was extracted by limited pepsin digestion [8] in collaboration with the National Institute of Immunology, New Delhi, India. Cells were resuspended in 67 mM PB, pH 5.8, to a final concentration of 1 g of bacteria in 2 ml of buffer. The cell suspension was warmed at 37°C and pepsin was added to a concentration of 1 mg/10 g of bacteria and incubated for 45 min at 37°C, with slow stirring. At the end of the process of digestion, the flask was transferred to an ice bath and solid sodium bicarbonate was added to raise the pH to c. 7.4. The bacteria were sedimented by centrifugation at 10,000 rpm for 20 min. Supernate was sterilised through a 0.2 µm filter (Millipore) and concentrated to c. one-fifth of its original volume by pressure filtration with a UM 10 membrane (Amicon Corp. Lexington, MA, USA). Concentrated protein was dialysed against 50 mM ammonium bicarbonate buffer and lyophilised.

Purification of M protein

Crude M protein was purified by DEAE Sephadex A-25 ion-exchange chromatography. Lyophilised crude M protein was dissolved in 10 mM PB, pH 8.0, loaded on a DEAE-Sephadex A-25 column (0.9 x 26 cm) and equilibrated with the same buffer. The protein was first eluted with the eluting buffer and then with a NaCl gradient 100 mM, 200 mM up to 1 M in 10 mM PB. Protein contents of the peaks were measured by Lowry's method [9]. Gel filtration was on a Sephadex G-200 (2.5 x 95 cm) column equilibrated and eluted with 10 mM ammonium bicarbonate buffer, pH 8.2. Column fractions were monitored for protein by measuring the absorbance at 280 nm.

SDS-PAGE

Peak fractions of ion-exchange chromatography were checked on polacrylamide gel 12% in the presence of SDS 0.1% with a Tris glycine buffer system [10]. Protein standards (14–66 kDa, Sigma) were run to enable estimation of the mol.wt.

Production of sera against whole-cell and M-protein fragments

M antisera were raised by whole-cell vaccination of rabbits. The bacteria were grown in normal human blood to check for the presence of M protein before preparation of the vaccine. Animals were immunised with 12 doses of whole-cell vaccine over 3 weeks. Antiserum to purified M protein (pep-MNT) was raised in rabbits; the primary inoculation consisted of 100 µg of M protein emulsified in Freund’s complete adjuvant (FCA), injected intracutaneously at multiple sites. A booster injection was given with the same dose of M protein emulsified in incomplete Freund’s adjuvant (IFA). Rabbits were bled at 2-week intervals for 8 weeks, to check for the development of antibodies.

Absorption of opsonic antibodies

Antiserum raised was checked for the presence of opsonic antibodies. Equal volumes of pep-M and antiserum raised against pep-M were mixed and incubated at 37°C for 1 h followed by incubation for 18 h at 4°C. The precipitate was sedimented by centrifugation at 3000 rpm for 20 min and the supernate was tested for its opsonic activity. Control absorption was done with comparable amounts of heterologous proteins.

Enzyme-linked immunosorbent assay

Enzyme-linked immunosorbent assay (ELISA) against known rheumatogenic M types and the M-non-typable strain was performed by the method described by Bisno and colleagues [11]. Microtitration plates were coated with 100 µl of purified M protein at a concentration of 10 µg/ml (1 µg/well/100 µl). Anti-M antibodies were detected in patients’ sera with horseradish peroxidase conjugated to rabbit anti-human IgG and 5 amino salicylic acid as a colour substrate. The titre of positive reactions were recorded as the dilution of...
serum that gave an absorption of >0.1 at 405 nm. Purified M proteins of known rheumatogenic M types (M5, M6, M18, M19 and M24) were kindly provided by Professor J.B. Dale, Memphis, Tennessee, USA.

**Indirect bactericidal test**

The assay was performed as described by Lancefield [12]. Heparinised whole blood from normal human donors was used as a source of phagocytes. Dilutions of specific M types of GAS (50 µl) were mixed with human blood (400 µl) in the presence or absence (control) of the test serum (100 µl) and rotated at 16 rpm at 37°C for 3 h. Survival of GAS was determined by the pour-plate method in the presence of sheep blood. Stationary and rotated controls without antisera were used to test the ability of the GAS to grow in donors' blood. Test runs were considered valid only when both the stationary and rotated controls exhibited complete haemolysis.

**Results**

**Isolation and purification of M protein**

The ion-exchange chromatography profile at 280 nm revealed seven major peaks of protein (Fig. 1). Proteins were dialysed against ammonium bicarbonate buffer, pH 8.0, followed by lyophilisation. Only peaks eluted with 200 mM NaCl in 10 mM PB showed immunoreactivity with the corresponding patients' serum and hyperimmune serum raised against whole-cell vaccine in rabbits.

**Purity check**

Peak fractions of protein were checked by SDS-PAGE along with the standard mol.wt markers, to estimate the mol.wt of proteins in peaks eluted with 200 mM NaCl in 10 mM PB that showed immunoreactivity. One peak was found to be homogeneous on SDS-PAGE, with a mol.wt 20 kDa while a second peak showed a major band at 20 kDa and minor low mol.wt bands. Other peaks showed multiple bands in the range 15–26 kDa.

**Gel filtration of immunoreactive peak**

The peak eluted from ion-exchange chromatography was further subjected to Sephadex G-200 gel filtration to separate the major protein from the lower mol.wt components present after the DEAE ion-exchange chromatography. However, the final yield of the purified protein, 14 mg/160 mg of crude protein, was very poor after gel filtration. The peak fractions after gel filtration were again checked for purity and estimation of the mol.wt by SDS-PAGE. The major peak showed a 20 kDa band.

**Immunological properties**

The immunoreactive peak fraction was studied for its ability to produce opsonic antibodies in rabbits. Rabbits were immunised with pep-MNT (mol.wt 20 kDa) by the method described by Manjula and Fischetti [8]. Hyperimmune serum showed lines of precipitation with corresponding crude M protein obtained after pepsinisation, as well as their corresponding pep-MNT fractions.

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**Fig. 1.** Ion-exchange chromatography of crude M protein on DEAE Sephadex (0.9 x 26 cm) in 10 mM PB, pH 8.0, and stepwise elution with gradient NaCl. Fraction volume 2.5 ml; flow rate 11 ml/h.
Evidence of opsonising antibodies in hyperimmune sera

The retention of the antigenic structure in pep-MNT was tested by determining its ability to absorb antibodies from hyperimmune serum in an indirect bactericidal assay [13]. Table 1 shows that unabsorbed serum against MNT was highly opsonic to type-specific streptococci. The opsonic activity of this serum was lost after absorption with soluble pep-MNT. This result indicates that the reduced phagocytosis observed after absorption was due to specific removal of type-specific opsonic antibodies.

Screening of RF, RHD and control sera for the presence of M antibodies against the 20-kDa M-protein fraction by ELISA

To study the prevalence of an M-non-typable strain in RF and RHD cases, type-specific antibody was measured by ELISA. The presence of antibodies against pep-MNT (20 kDa) was first checked in standard antisera (M1, M3, M5, M6, M18, M19 and M24) obtained from Dr E.L. Kaplan, Minnesota, USA, along with the serum raised against the 20-kDa fragment of M protein. Corresponding hyperimmune serum showed positive titres up to a 1 in 128,000 dilution; other standard M antisera did not, even at 1 in 100 dilution. This indicates that pep-MNT is highly specific.

Sera from 400 patients (200 each of RF and RHD) and 75 controls were screened for type-specific antibodies against pep-MNT by ELISA. A titre of 1 in 800 was considered positive for type specific antibodies.

The opsonisation-inhibition assay was performed to confirm the type-specific antibody in ELISA-positive sera (1 in 400 and 1 in 800). Only five positive sera were checked and four sera were found to be positive for type-specific antibodies; this correlated well with ELISA results (Table 4).

Screening of RF and control sera for the presence of M antibodies against purified M proteins of known rheumatogenic M types by ELISA

Out of 150 sera titrated for antibodies to known rheumatogenic types, only 24 sera (16%) showed significant levels of antibodies against types M5, M6, M18, M19 and M24. However, of 24 positive sera, 14 (58.3%) showed multiple antibodies. Antibodies against individual M proteins were as follows: six (4%) RF patients were positive for M-type 5 antibodies, three (2%) for M-type 6, six (4%) for M-type 18, seven (4.6%) for M-type 19 and 12 (8%) for M-type 24. Of these, type 24 was predominant. Among control sera only one subject had antibodies to types M6 and M19 at a titre of 1 in 800. Results are presented in Tables 5 and 6.

In the sera of nine RF patients who yielded GAS, seven had antibodies against known rheumatogenic M types, but none yielded known rheumatogenic M types from throat culture.
Table 3. Antibodies against 20-kDa M-protein fragment of MNT strain in paired RF sera

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Serum phase</th>
<th>Antibody titre</th>
<th>Serological activity (ASO)</th>
<th>Details of GAS isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>I</td>
<td>&lt; 100</td>
<td>+</td>
<td>T2, SOF-ve, M-5</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>&lt; 100</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>I</td>
<td>&lt; 200</td>
<td>-</td>
<td>TNT, SOF-ve, MNT</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>200</td>
<td>+</td>
<td>3/13/B3264, SOF-ve,</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
<td>200</td>
<td>+</td>
<td>5/12/27, SOF-ve, MNT</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>&lt; 100</td>
<td>-</td>
<td>3/13/B3264, SOF-ve, MNT</td>
</tr>
<tr>
<td>4</td>
<td>I</td>
<td>200</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>&lt; 100</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>I</td>
<td>100</td>
<td>+</td>
<td>T2, SOF-ve, MNT</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>400</td>
<td>+</td>
<td></td>
</tr>
<tr>
<td>6</td>
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<td>&lt; 100</td>
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<td>3/13/B3264, SOF-ve, MNT</td>
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<tr>
<td></td>
<td>II</td>
<td>100</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>7</td>
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<td>&lt; 100</td>
<td>+</td>
<td>T2, SOF-ve, MNT</td>
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<td>+</td>
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<td>8</td>
<td>I</td>
<td>&lt; 100</td>
<td>+</td>
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<td>II</td>
<td>&lt; 100</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>I</td>
<td>&lt; 100</td>
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<td>TNT, SOF-ve, MNT</td>
</tr>
<tr>
<td></td>
<td>II</td>
<td>&lt; 100</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Opsonisation-inhibition assay for presence of opsonic antibody in ELISA-positive sera against 20-kDa M-protein fragment of MNT strain

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Number of sera positive by ELISA</th>
<th>Number of sera tested</th>
<th>Number (%) positive</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF and RHD</td>
<td>40</td>
<td>400</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>800</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1600</td>
<td>2</td>
</tr>
<tr>
<td>Controls</td>
<td>1</td>
<td>400</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>800</td>
<td>...</td>
</tr>
<tr>
<td></td>
<td>1600</td>
<td>...</td>
<td>...</td>
</tr>
</tbody>
</table>

Table 5. Anti-M antibodies against known rheumatogenic types in RF and control subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Dilution of serum</th>
<th>Number of patients positive for antibodies to M types</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 in 400</td>
<td>M5</td>
</tr>
<tr>
<td>RF (150)</td>
<td>22</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>3</td>
</tr>
<tr>
<td>Controls</td>
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<td>3</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

An OD >0.1 at a 1 in 800 dilution was considered positive for specific antibodies.

Discussion

This study aimed to identify predominant serotypes of GAS responsible for RF and RHD in India. Existing evidence [14–20] suggests that individual strains of GAS may vary in their ability to produce RF and only a limited number of M types have been associated epidemiologically with RF [4,21]. The frequency of isolation of GAS from throat cultures in RF cases is very poor because of the latent period between the upper respiratory tract infection and onset of RF. Therefore, an alternative approach was employed by...
the assay of type-specific antibodies by ELISA to potentially rheumatogenic M types in the serum of RF patients.

Of 200 RF cases screened for type-specific antibodies, nine RF patients yielded GAS. Of these, four were isolated in a short period of 28–42 days. All these four strains were SOF-negative and M-non-typable in this laboratory; this was confirmed by Dr Diana Martin, (New Zealand) and Dr E.L. Kaplan (Minnesota, USA). Hyperimmune serum raised against these strains cross-reacted with these other strains. As all four strains seemed to belong to a new provisional M type, it was decided to purify M protein from one of them and to use this to screen for M antibodies in RF and RHD patients.

M protein was purified by loading crude M protein in DEAE-Sephadex A-25 ion-exchange chromatography. The chromatographic behaviour of pep-MNT showed that it was adsorbed strongly to gel equilibrated with 10 mM PB, pH 8.0, and eluted with NaCl gradient in PB (100 mM–1 M).

Only peaks eluted with 200 mM NaCl in 10 mM PB, pH 8.0, retained their immunological properties, i.e. (i) line of precipitation with corresponding serum and absorbed type-specific hyperimmune serum produced in rabbits; (ii) retention of antigenic structure capable of neutralising the opsonic activity of the antiserum raised against whole-cell antigen; (iii) immunogenicity in rabbits producing protective opsonic antibodies.

Pep-M showed a protective antigenic structure in the 20 kDa fragment of M protein and was used as an antigen for ELISA. To examine the prevalence of M antibodies against this 20-kDa pep-M of the M-non-typable strain, a total of 400 RF and RHD sera was screened. The type-specific antibodies were found in 9% of RF and 4% of RHD cases. However, of the sera from 75 normal healthy age-matched controls, only one (1.33%) showed the presence of antibodies at a significant titre. Statistical analysis showed that prevalence of MNT antibodies was significantly higher in patients than in controls.

Prevalence of M antibodies against known rheumatogenic M types 5, 6, 18, 19, 24 isolated from outbreaks in different countries were observed in 2–6% of cases of RF. Most of the cases had antibodies against more than one M type. However, M-type 24 antibodies were predominant, singly as well as in association with other M antibodies. The presence of multiple antibodies in 58.3% of sera against known rheumatogenic M types could be explained either by the fact that the patient had been exposed to these M types or that these was some cross-reactivity due to the presence of a common epitope in certain M types. These data support the concept of Beachey et al. [22, 23] that each M protein may contain several type-specific determinants among many subpeptides of the isolated M molecule.

These results show that known rheumatogenic M types are prevalent in India and play a role in the development of RF and RHD. Prevalence of M antibodies against pep-MNT compared with that of M antibodies against known rheumatogenic M types showed that the prevalence of the MNT strain is higher than that of known rheumatogenic M types in an Indian community. Higher prevalence of the MNT strain indicates that an outbreak of RF could be possible with this provisional new M type. The protective determinant of this M type appears to be a 20-kDa M-protein fragment, so this could be a future candidate for the preparation of a synthetic multivalent M-protein vaccine.

We thank Professor J.B. Dale, VA Medical centre, Memphis, TN, USA, for continuous support and suggestions. This study was supported by grants under the Indo-US project from National Institute of Health, Bethesda, MD, USA.

References


