Research Article

Evaluation of anti-bacterial IgG antibodies among rheumatoid arthritis and non rheumatoid arthritis patients with special reference to anti Proteus antibodies

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ABSTRACT

Background: The objective of the study was to detect IgG antibodies against commonly isolated bacterial species among rheumatoid arthritis (RA) and non rheumatoid arthritis patients sera with special reference to anti Proteus antibodies.

Methods: Fifty each of Rheumatoid factor positive, Rheumatoid factor negative, C reactive protein positive and C reactive protein negative sera were tested for IgG antibodies against ‘O’ antigens of Proteus mirabilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi and Pseudomonas aeruginosa by indirect ELISA method using inactivated whole cells as the antigen.

Results: Anti Proteus antibodies were detected in more number of (29/50) RA patients than non RA patients. The antibodies to other bacteria were detected in less number of sera. More number of C reactive protein positive sera was showing IgG antibodies to several bacterial pathogens than C reactive protein negative sera.

Conclusions: Elevated levels of anti Proteus antibodies among RA patients clearly demonstrates the role of infectious agents in diseases like RA but it is not clear whether this role is a cause or effect. Further studies with large series at multiple centers may widen the treatment options for RA in future.

Keywords: Rheumatoid arthritis, Anti-proteus antibodies, Autoimmune disease

INTRODUCTION

Rheumatoid arthritis (RA) is a relatively common (0.5% - 1%) chronic inflammatory systemic disease that primarily affects peripheral joints. Its clinical course is characterized by insidious progression and destruction of the affected joints, resulting in significant morbidity and mortality. Despite considerable efforts to ascertain its cause, the etiology of RA remains an enigma. Extensive investigations of metabolic, endocrine, nutritional, as well as psychosocial, geographic, ethnic and occupational factors suggests that some or all may influence the course of the disease but there is very little evidence that they are involved in its cause.

In recent years, research in this area has concentrated on the interplay between genetic susceptibility and environmental exposure. RA associated HLA molecules that stem from the HLA-DR4 locus has been considered to be genetic markers for disease evolution. In patients with RA, certain HLA-DR4 subtypes i.e., Dw4, Dw10, Dw14 and Dw15 predominate.²
Although much remains uncertain, it is currently believed that RA is triggered by exposure of an immunogenetically susceptible host to an arthritogenic microbial antigen. Bacterial and viral infectious agents have been shown to produce polyarthritis, often resembling RA, in animals and humans. Those that have been implicated include Cytomegalovirus, Parvovirus, Rabella virus, Epstein-Barr virus, Staphylococci, Streptococci, Mycoplasma arthritis, Mycobacterium tuberculosis and others. One microorganism, which many investigators have linked with RA, is Proteus mirabilis, the organism that after Escherichia coli is the most frequently associated with urinary tract infections.

Several studies were conducted to know the incidence of anti Proteus mirabilis antibodies in RA patients by using either tube agglutination method or ELISA technique. The control groups in the study were variable and also the antigen used by them was either particulate type or sonicated type from the Proteus mirabilis cultures. Some people used a particular strain of Proteus mirabilis (B 17) and used higher number of cells (1.57 X 10^12 cells / ml) for getting optimal agglutination by indirect agglutination method using coomb’s serum. Several workers used ELISA technique with SDS extracts of wild strains of Proteus mirabilis and Escherichia coli and standard strains of Klebsiella pneumoniae like 21, 43 & ATCC 27736. Some workers performed simple tube agglutination test but they did not mention the method of standardizing the antigen.

Anti Proteus antibodies (APA) could be demonstrated in serum, urine as well as in jejunal fluids in several studies. However the studies did not indicate the relationship between Rheumatoid factor (RF) and the incidence of anti Proteus mirabilis antibodies.

So it has been decided to study the presence of antibodies against Proteus mirabilis and also against commonly isolated bacteria like Escherichia coli, Klebsiella pneumoniae, Salmonella typhi and Pseudomonas aeruginosa in RF positive, RF negative, C-reactive protein (CRP) positive and CRP negative serum samples as CRP indicates either ongoing infection or an acute/chronic inflammation.

**METHODS**

Fifty serum samples obtained from patients clinically diagnosed as RA based on 1987 revised criteria for RA by the American Rheumatology Association who were RF IgM positive and fifty serum samples obtained from non-RA patients (Osteoarthritis, Fibromyalgia, mechanical injuries, nonspecific arthritis etc) who were RF IgM negative were tested.

Fifty serum samples, which were CRP positive, and fifty serum samples, which were CRP negative, were also tested.

The bacterial cultures tested in the study were wild strains isolated from urine specimens of patients attending our hospital. All the isolates were identified by standard methods and were stored on Brain heart infusion agar (BHI) slants for further studies. The following bacterial isolates were studied – Proteus mirabilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi and Pseudomonas aeruginosa.

RF IgM positivity was determined by ELISA method (BL DIAGNOSTIKA, Germany) and CRP positivity was determined by Immunoturbidimetry (QUANTA CRP, TULIP DIAGNOSTICS). The manufacturer’s instructions were followed for the interpretation of the results obtained.

All the serum samples were tested for antibodies to ‘O’ antigens of Proteus mirabilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi and Pseudomonas aeruginosa by indirect micro ELISA.

**Elisa technique**

**Preparation of antigen**

- The preserved cultures of Proteus mirabilis, Escherichia coli, Salmonella typhi and Pseudomonas aeruginosa on BHI agar slants were sub cultured in BHI broth. After 24 hours incubation the cultures were then inoculated onto freshly prepared plates of BHI agar containing 1:800 phenol except for Klebsiella pneumoniae, which was inoculated onto BHI agar devoid of phenol.
- After 24 hours of incubation, the growth on the plate was scraped gently with a sterile loop and suspended in 3 ml of sterile normal saline (0.9% w/v). The organisms were then heat killed by maintaining the culture suspension in a water bath at 60°C for 30 minutes.
- The culture suspension was then centrifuged at 3000 RPM for 15 minutes. The supernatant was discarded and the pellet was washed with 1000 μl of phosphate buffered saline (PBS) pH 7.2 for 3 times.
- After the third wash, carbonate-bicarbonate buffer (pH 9.6) was added to the pellet slowly, and the turbidity was adjusted using McFarland turbidity standard tubes so that the antigen suspension contains 109 organisms / ml.

**Antigen coating of plates**

- 100 μl of antigen suspension was added to each well of Immunomaxi ELISA plate (Axygen Scientific Pvt.Ltd). The plate was covered with aluminum foil and incubated at 37°C for 3 hours for sensitization. The sensitized plate was then kept at 4°C over night.
- The plate was washed 3 times using 400 μl of wash buffer per well. (PBS-T. 1% Tween 20 in 0.15 M PBS, pH 7.2)
• Blocking was done with 150 μl of 1% bovine serum albumin (BSA) solution and incubating the plate at 37°C for 2 hours.
• The plate was again washed 3 times as described above.

**ELISA Procedure**

• 100 μl of RF positive, RF negative, CRP positive and CRP negative serum samples diluted 1:10 using PBS-T were added to each well and incubated for 90 minutes at 37°C.
• After thoroughly washing the plate thrice with PBS-T, 100 μl of 1:10,000 diluted Rabbit anti-human IgG conjugated to Horse Radish Peroxidase was added and incubated again at 37°C for 90 minutes.
• The plate was washed 3 times again and reaction was developed by adding 100 μl of Chromogenic substrate (5 mg OPD in 10 ml Phosphate citrate buffer, pH 5.0 containing 10 μl of 30% H₂O₂) and incubating at 37°C for 20 minutes.
• The reaction was stopped with 50 μl of 3M H₂SO₄ and absorbance was measured at 492 nm in an ELISA reader.
• The calculated ELISA titres were expressed as the mean OD±2 SD for each organism tested.

**RESULTS**

A total of 50 RF positive, 50 RF negative, 50 CRP positive and 50 CRP negative sera were tested for the presence of IgG antibodies against commonly isolated species of Enterobacteriaceae and *Pseudomonas aeruginosa* by indirect ELISA method.

**Table 1: Sex distribution of the sera tested.**

<table>
<thead>
<tr>
<th>Serum</th>
<th>Total no. tested</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF IgM Positive</td>
<td>50</td>
<td>15</td>
<td>35</td>
</tr>
<tr>
<td>CRP Positive</td>
<td>50</td>
<td>34</td>
<td>16</td>
</tr>
<tr>
<td>CRP Negative</td>
<td>50</td>
<td>25</td>
<td>25</td>
</tr>
</tbody>
</table>

Table 1 shows sex distribution of the serum samples tested. More number of RF positive patients was females as incidence of RA is more among them.

**Table 2: Presence of Antibacterial IgG antibodies in RF positive & RF negative sera.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>RF positive sera</th>
<th>RF negative sera</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. positive</td>
<td>Percent</td>
</tr>
<tr>
<td>Proteus mirabilis</td>
<td>29</td>
<td>58</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>04</td>
<td>08</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>02</td>
<td>04</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>03</td>
<td>06</td>
</tr>
</tbody>
</table>

Among RF negative sera 06 (12%) were positive for anti *Salmonella* antibodies followed by anti *Proteus* antibodies in 02 (04%) and anti *Pseudomonas* antibodies in 01 (02%) patient. None of the RF negative sera showed any IgG antibodies against *Klebsiella pneumoniae* and *Escherichia coli* antigens.

**Table 3: Presence of Anti bacterial IgG antibodies in CRP positive & CRP negative sera.**

<table>
<thead>
<tr>
<th>Organism</th>
<th>CRP positive</th>
<th>CRP negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Proteus mirabilis</td>
<td>10</td>
<td>20</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>04</td>
<td>08</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>08</td>
<td>16</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>12</td>
<td>24</td>
</tr>
<tr>
<td>Pseudomonas aeruginosa</td>
<td>06</td>
<td>12</td>
</tr>
</tbody>
</table>

Table 3 shows the presence of IgG antibodies in CRP positive and CRP negative sera. Among CRP positive, 10 (20%) were positive for anti *Proteus* antibodies followed by anti *Salmonella* antibodies in 12 (24%), anti *E.coli* in 08 (16%), anti *Klebsiella* in 04 (08%) and anti *Pseudomonas* in 06 (12%) patients. In CRP negative sera only two patients showed IgG antibodies against *Escherichia coli* and *Salmonella typhi*.

**DISCUSSION**

Chronic inflammatory diseases of autoimmune origin may have exacerbations and remissions in the clinical symptoms due to multiple environmental factors.
Infectious agents may play an important role in triggering the immune response in these patients and may produce exacerbations. Although it is still controversial, several infectious agents have been implicated to play an important role in the diseases like RA but it is not yet clear whether their role is a cause or effect.\textsuperscript{4,8}

In the present study, we have attempted to determine antibodies against Proteus mirabilis and also against commonly isolated bacteria like Escherichia coli, Klebsiella pneumoniae, Salmonella typhi and Pseudomonas aeruginosa using inactivated whole cells as the antigen. Among the 50 RF positive sera that were tested, anti Proteus antibodies of IgG type were found in 29 (58\%) sera by indirect ELISA technique. The antibodies to other bacteria tested were detected in less number of sera. The mean antibody titres (± 2SD) against Proteus mirabilis whole cell antigen in RF positive sera was 1.74 ± 0.6 and this was significantly higher than the RF negative sera which was 0.13 ± 0.3 (p < 0.001) (Table 4). Senior et al demonstrated all classes of antibodies (IgG, IgA & IgM) against Proteus antigen in urine by ELISA technique.\textsuperscript{13} Chou et al studied 39 sera from RA patients and 52 sera from Ankylosing spondylitis patients for the presence of IgG antibodies against Proteus mirabilis and found that they were elevated in RA patients.\textsuperscript{15} Some studies from our country described the presence of anti Proteus mirabilis antibodies in about 70 to 80\% of cases of RA by tube agglutination method.\textsuperscript{8,10} The high incidence of anti Proteus antibodies in these studies could be due to the fact that tube agglutination method was used, that detects total antibody levels, which can give rise to high incidence of antibody positivity. Further these authors did not mention about the standardization of the bacterial suspension used in their studies.

Table 4: Antibody titres of Proteus mirabilis in various sera (Mean ± 2 SD).

<table>
<thead>
<tr>
<th>Serum</th>
<th>Number</th>
<th>Antibody titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF Positive</td>
<td>50</td>
<td>1.74 ± 0.6</td>
</tr>
<tr>
<td>RF Negative</td>
<td>50</td>
<td>0.13 ± 0.3</td>
</tr>
<tr>
<td>CRP Positive</td>
<td>50</td>
<td>1.56 ± 0.7</td>
</tr>
<tr>
<td>CRP Negative</td>
<td>50</td>
<td>0.09 ± 0.4</td>
</tr>
</tbody>
</table>

From the above discussion it is clear that anti Proteus antibodies of all classes can be detected in RA patients depending upon the stage of the disease. Difference in antibody positivity incidence showed by different authors might be due to difference in techniques employed for the detection of antibodies like tube agglutination method, indirect immunofluorescence, indirect ELISA technique and also different types of antigen preparations used (Sonicated antigen or SDS treated bacteria or whole bacterial cells live or killed).\textsuperscript{6,10,12,16} In our study, we used killed bacteria suspended in carbonate-bicarbonate buffer. Further in our study the cut off value taken was mean ± 2 SD above to increase the specificity, where as some authors used mean ± SD only.\textsuperscript{10,16} This also might have contributed to the variation in the results of various studies.

In the present study antibodies were also detected against commonly isolated Enterobacteriaceae isolates and Pseudomonas aeruginosa in RF positive sera but to a lesser extent. However in RF negative sera the incidence of antibody positivity was very low except for Salmonella typhi, which was positive in 06(12\%) cases. This observation supports the gut translocation of bacteria and the resulting immune response might be proportional to the number of cells translocated or it could be due to cross-reactive antibodies.

This finding of raised antibodies against Proteus mirabilis suggests that RA could be a form of reactive arthritis caused by sub clinical infection more frequently with Proteus mirabilis. There are several ways in which infection could lead to activation of an autoimmune response, including the initiation of tissue damage leading to the exposure of previously hidden self-antigens or the elaboration of super antigens. It has been proposed that similarities in antigenic proteins between infecting pathogens and host tissues might result in the immune response against the pathogen being misdirected against the host tissue.\textsuperscript{17} The evidence provided in support of this mechanism is the finding of homology between the susceptibility sequence (EQRRAA) on HLA DR B1 0401 of RA and Proteus mirabilis haemolysin (ESRRAL), and the finding of similarity between type XI collagen (LRRE1) and Proteus mirabilis urease (IRRET).\textsuperscript{18}

Another interesting finding in our study was more number of CRP positive sera (CRP > 10mg / L) was positive for IgG antibodies to several bacterial pathogens like Proteus mirabilis (20\%), Klebsiella pneumoniae (08\%), Escherichia coli (16\%), Salmonella typhi (24\%) and Pseudomonas aeruginosa (12\%). High levels of CRP indicate either inflammation or infection due to bacteria or fungi but not viruses. Presence of antibodies to commonly isolated bacteria in CRP positive sera indicates that either inflammation due to immune complexes or infection due to one of the bacterial species.\textsuperscript{19} This antibody response further aggravates the condition by stimulating various mediators like cytokines, complement and other vaso active substances or inflammatory responses. Most of the patients with active autoimmune diseases like RA, Systemic Lupus Erythematosus (SLE) etc show elevated CRP which can be due to an active focus of infection. So these patients should be investigated for any infective focus due to common bacterial pathogens and treated accordingly.

CONCLUSIONS

In conclusion, viewing RA as a form of reactive arthritis is an appealing hypothesis because it holds the promise of treatment and even prevention of disease. Further more, the hypothesis provides a conceptual link between the
dual roles of the immune response as a physiologic defense against foreign organisms and as a mediator of pathologic autoimmunity. Data has recently emerged on the beneficial effects of minocycline in RA but the mechanism of action is unknown, and may as likely be anti-inflammatory as anti-microbial. More over, the present knowledge regarding detection of anti Proteus antibodies in patients with RA has several gaps with respect to the technique to be used and standardization of the antigen. Further studies should give importance to these lacunae and continuous efforts are required to demonstrate even more rigorous proof in the quest to link infection and RA. The presence of antibodies to commonly isolated gram-negative bacteria in CRP positive sera suggests either inflammation due to immune complexes or infection and justifies the use of broad spectrum antibiotics in the absence of evidence for any active infection in the form of culture positivity if the patient is symptomatic.

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Ethical approval: The study was approved by the Institutional Ethics Committee

REFERENCES