Original article

Cell-wall-deficient bacteria: a major etiological factor for psoriasis?

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Keywords: cell-wall-deficient bacteria; psoriasis; interferon-γ; T lymphocytes proliferation; antibiotic therapy

Background  Psoriasis is a common inflammatory skin disease, yet knowledge of the factors that may induce, trigger, or exacerbate psoriasis is not fully delineated. Recent advances have improved our understanding of the link between psoriasis and cell-wall-deficient bacteria (CWDB) infections. In the present study we assessed the prevalence of CWDB infection in patients with psoriasis.

Methods  The carriage rate of CWDB in the tonsil or pharynx of psoriasis patients, chronic tonsillitis patients and controls were investigated using hypertonic medium. Psoriasis patients with CWDB were randomly assigned to two groups and respectively treated with antibiotics or systemic therapy without antibiotic. Human peripheral blood mononuclear cells (PBMC) from psoriasis patients, chronic tonsillitis patients and control subjects were stimulated with bacteria antigens and extra-cellular levels of interferon-γ (IFN-γ) and interleukin (IL)-10 were measured in the supernatants using the ELISA technique, in vitro. Meanwhile, the proliferation ability of PBMC to respond to bacteria antigens was detected by MTT assay.

Results  CWDB were isolated from 74.2% of psoriasis patients, 23.5% of chronic tonsillitis patients and only 6.3% of controls. Antibiotic therapy was appropriate for approximately 80% of psoriasis patients with CWDB infection, and in only 8.9% psoriasis patients CWDB infection was detected after antibiotic therapy. Meanwhile, our study showed that CWDB and wide-type bacteria did remarkably enhance the production of IFN-γ, in vitro, and PBMC proliferation.

Conclusion  CWDB infection may be a virtual triggering factor in psoriasis by regulating T-cell activation.

Psoriasis is a common inflammatory skin disease that causes scaly pink patches, affecting an estimated about 2% of the world population. The two most common clinical forms of the disease are guttate psoriasis and chronic plaque psoriasis in Chinese population. Guttate psoriasis is a distinctive acute form of psoriasis, and typically erupts explosively over large areas of the skin surface, usually 1–2 weeks after an episode of acute tonsillitis or pharyngitis. If left untreated, guttate psoriasis may clear spontaneously after a period of months or may develop into chronic plaque psoriasis.

Knowledge of the factors that may induce, trigger, or exacerbate psoriasis is of primary importance in clinical practice. Extensive evidence supports that the disease can be provoked or exacerbated by a variety of different environmental factors, particularly Streptococcus pyogenes, which has been recognized for at least 50 years and implicated in both acute and chronic forms of the disease. The link between psoriasis and infections is probably explained by the “superantigen theory”, that superantigens are the products of bacteria, virus or fungi, which can bypass normal immunological pathway and cause powerful stimulation of the immune system. The nature of the psoriatic antigens, however, remains unknown. Earlier studies imply that a protein called the M-protein carried by the streptococcus acts as a superantigen in provoking or exacerbating of psoriasis.

Recently it has been reported that peptidoglycan is a major etiological factor for psoriasis and its importance of peptidoglycan in the bacterial-infection-induced inflammatory disease is emphasized. In view of the associated bacterial infection, some dermatologists recommend the use of antibiotic therapy in psoriasis patients. Although some studies have shown that macrolides could be used as one of the adjunctive therapies for psoriasis vulgaris, in other reports antistreptococcal intervention was not beneficial for guttate psoriasis and chronic plaque psoriasis.

Hence, there are conflicting views in the literature regarding the triggering infection factors and the efficacy of antibiotics on psoriasis. We think that the evidence of new kind infection factor in psoriasis needs further studies and evaluation. Cell-wall-deficient bacteria

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(CWDB) are pleomorphic bacterial forms, which can pass through 0.22 µm filters. Recent advance has implied that CWDB can upregulate to a more aggressive pathological form.9

In this study, we aimed to test the hypothesis that the triggering of psoriasis is mediated by CWDB.

METHODS

Patient selection
Psoriasis patients and chronic tonsillitis patients, who were admitted to Weihai Municipal Hospital affiliated to Dalian Medical University, were studied during the period June 2007–June 2009. A total of 124 psoriasis patients aged between 19 and 55 years, were enrolled in the study, including 65 guttae psoriasis and 59 chronic plaque psoriasis. Severity of the disease was scored as per “Psoriasis Area and Severity Index” (PASI) scoring system. Patients who had used any topical or systemic therapy for psoriasis or systemic antibiotics during the preceding 4 weeks were excluded from the study. The study consisted of 81 patients who were diagnosed as having chronic tonsillitis on the basis of history, throat culture, and clinical examinations. They were healthy otherwise. The cases with use of systemic antibiotics during the preceding 4 weeks were excluded. A total of 79 control subjects were taken by random-digit telephone dialing to find. Individuals with history of sore throat or use of systemic antibiotics during the preceding 4 weeks were excluded. Demographic profiles were noted including age and gender of the patients and controls.

Human subjects assurances
Informed consent for the study was obtained, at the time patients and healthy controls were enrolled in our program. The primary protocol, the CWDB substudy, and all other treatments in the program were reviewed and approved by local institutional academy.

CWDB culture
Pharyngeal specimen collection and CWDB culture
A pharyngeal specimen from each patient was obtained by swabbing both tonsillar surfaces and the posterior pharynx with a sterile cotton-tipped swab, as well as controls. After the 4-week treatment period of psoriasis patients, it was repeated.

The swabs and quality control standard were sent to the microbiology laboratory where they were mixed in 0.5 ml 0.9% NaCl. The solution was filtered through 0.22 µm filter and added to 2 ml hypertonic CWDB liquid medium for the induction and growth of CWDB. It was incubated at 37°C in 5% carbon dioxide.

CWDB reverting, identification and antimicrobial susceptibility
On day 14, 20 µl of the broth was subcultured on hypertonic CWDB solid medium plate, incubated at 37°C in 5% carbon dioxide for 5 days. Cultivation of CWDB was reverted to wild-type bacteria using regular in vitro subcultures on 5% sheep blood agar plate. Wild-type bacteria were identified independently by the Vitek System (BioMerieux Vitek, USA). The antimicrobial susceptibility of wild-type bacteria was tested by the Kirby Bauer disk-diffusion method and results were interpreted according to the Clinical and Laboratory Standards Institute (CLSI) criteria.

During CWDB culture and reversion, observation of CWDB colony forms and Gram staining were performed to distinguish CWDB and wild-type bacteria by CWDB typical colony forms and distinctive polymorphic forms.

Quality control procedures of culture
Blood agar plates were incubated and examined for 1 week. A sterile cotton-tip of CWDB culture was performed regularly to check for gross bacterial contamination. All laboratory personnel were blinded to the case or control status of individual slides.

Influence of CWDB on peripheral blood mononuclear cells (PBMC)
Preparation of bacterial antigens and PBMC
Twenty samples of CWDB isolated from patients with psoriasis were mixed for the preparation of bacterial antigens, as well as wild-type bacteria. They were harvested in liquid sodium and washed twice with PBS by centrifugation (7000 ×g, 15 minutes, 4°C). These bacteria mixtures then were subjected to 6 cycles of freeze (–70°C, 30 minutes)-thaw (37°C, 15 minutes) cycles, after adjusted to the same optical density (OD) with PBS. The solutions were filtered through 0.22 µm filter.

PBMC were collected by density gradient centrifugation from psoriasis patients, chronic tonsillitis patients, and control subjects admitted to our hospital and participated in this study. PBMC were suspended in RPMI-1640 medium containing 20% fetal calf serum and studied cell culture and MTT assay was performed immediately.

Cells culture and determination of cytokines production
PBMC (4×10^5/ml) and bacterial antigens were added at a ratio of 1:1 (v/v) in 96-well-flated bottom microplates, incubated at 37°C with 5% carbon dioxide. Cells were also cultured with phytohemagglutinin (PHA 15 µg/ml) and PBS, respectively. After incubation for 48 hours, the culture plates were centrifuged and supernatants were collected to determine extracellular IFN-γ and IL-10, using ELISA kits from Bender MedSystems (Vienna, Austria) according to the manufacturer’s directions. Measurements were made from triple samples, and the experiment was performed twice.

MTT assay
Bacterial antigens, PHA and PBS were respectively added at a ratio of 1:1 (v/v) to mononuclear cells (4×10^7/ml) in 96-well-flat bottom microplates at 37°C with 5% carbon dioxide. After incubation for 48 hours, the MTT
solution (1 mg/ml; Sigma, St. Louis, MO, USA) was added at 1:2 volume of culture medium for 1 hour at 37°C. The solution absorbance was measured by a microplate reader using test wavelength of 570 nm and reference wavelength of 630 nm.

**Antibiotic therapy of psoriasis patient with CWDB infection**

The patients’ baseline scores on the Psoriasis Area and Severity Index (PASI) were calculated. The patients were assigned into two treatment groups based on their PASI, with the first patient's treatment group randomly chosen, then the next patient with a similar PASI placed in the other group. Group A (45 patients) were treated only with antibiotic therapy for 4 weeks according to the antibiotics susceptibility and the characteristics of CWDB. Group B (47 patients) were given emollients for topical therapy and narrow-band ultraviolet B phototherapy. The physician’s global assessment (PGA) was made after 4-week treatment using the following grading: remission, marked improvement, improvement, no change and worse.10

**Statistical analyses**

Differences of age and gender among the three groups were assessed using one-way analysis of variance (ANOVA) and chi-square test, respectively. For comparisons of different PASI between the group A and group B, paired-sample t test was employed. Differences of CWDB carriage rate in the tonsil or pharynx among psoriasis patients, chronic tonsillitis patients and control were compared using Chi-square test. Comparisons of cytokines production and PBMC proliferation among three groups were respectively made by one-way ANOVA. Statistical analysis was performed using the SPSS12.0.1 statistical software package (SPSS, Inc., USA). *P* < 0.05 was considered as statistically significant.

**RESULTS**

**Descriptive results**

There were no statistically significant difference in the mean age and gender between the psoriasis patient group, chronic tonsillitis group and control group (*P* = 0.065 and 0.071). No statistically significant differences were found between the mean PASI of the two groups (*P* = 0.174).

**CWDB culture of pharyngeal specimen**

A total of 376 pharyngeal specimens were evaluated for the presence or absence of CWDB. CWDB culture of 74.2% (92/124) psoriasis patients exhibited positive and 23.5% (19/81) chronic tonsillitis were positive. Only the cultures of 6.3% (5/79) control subjects were positive. There was a statistically significant difference between psoriasis patients group, chronic tonsillitis patients groups and control groups (*P* < 0.001). CWDB isolates were only 8.9% (4/45) positive in psoriasis patients of group A after therapy but 53.2% (25/47) positive in psoriasis patients of group B.

The Gram staining appearance of a Gram-negative isolate recovered for CWDB forms from psoriasis are shown in Figure. Pleomorphic Gram-negative bacteria grew on hypertonic CWDB solid medium plates. Variations of the bacterial cells were evident, both in the size and the morphology of the individual cells; the third subcultured revenants from the colony on 5% sheep blood agar plate were pleomorphic, showing mostly gram-negative cell-wall-deficient forms with a minority of normal cell walls; the fifth subcultured revenants from the colony on 5% sheep blood agar plate were normal cell walls and subsequently identified to *Acinetobacter lwoffii*.

In psoriasis patients, 79.3% (73/92) of the predominant morphotype were Gram-negative bacilli, compared with Gram-positive cocci that were 20.7% (19/92). Non-fermenters Gram-negative bacilli represented the main Gram-negative bacteria involved in psoriasis. Head of the list was *Chryseemonas luteola* (20.5%), followed by *Burkholderia cepacia* and *Enterobacter cloacae*. Most Gram-negative revenants isolated from psoriasis patients are still susceptible to ciprofloxacin (100%), amikacin (100%), levofloxacin (100%) and gentamicin (100%). Most Gram-positive revenants of CWDB from the pharyngeal specimen of psoriasis patients were *Streptococcus pyogenes*. The highest sensitivity in
gram-positive revenants was observed for ciprofloxacin (100%), levofloxacin (100%) and tetracycline (100%).

**Influence of CWDB on PBMC**

Cells culture and determination of cytokines production
PBMC from psoriasis patients, chronic tonsillitis patients and control subjects were stimulated with bacteria antigens for 48 hours. Extra-cellular levels of IFN-γ and IL-10 were measured in the supernatants using the ELISA technique. As shown in Table 1, there were striking differences of extra-cellular IFN-γ levels between PBS and other antigens stimulation for PBMC (*P* <0.001); whereas, similar results for extra-cellular IFN-γ with CWDB antigens and wild-type bacteria antigens were obtained among psoriasis patients, chronic tonsillitis patients and control subjects (*P* =0.671 and 0.258). IL-10 levels in the supernatant of wild-type bacteria antigens and CWDB antigens stimulation cell culture were low, and no differences were observed among psoriasis patients, chronic tonsillitis patients and control subjects (*P* =0.329, Table 2).

### Table 1. Comparison of IFN-γ levels in mononuclear cell culture supernatants with the bacteria antigen stimulation among psoriasis patients, chronic tonsillitis patients and control subjects (pg/ml)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>CWDB</th>
<th>Wild-type bacteria</th>
<th>PBS</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psoriasis patients</td>
<td>10</td>
<td>9.45±0.91</td>
<td>9.42±1.45</td>
<td>5.14±0.69</td>
<td>10.39±1.40</td>
</tr>
<tr>
<td>Chronic tonsillitis</td>
<td>10</td>
<td>9.13±1.25</td>
<td>9.01±1.29</td>
<td>5.07±0.72</td>
<td>11.16±1.94</td>
</tr>
<tr>
<td>Control subjects</td>
<td>10</td>
<td>9.31±1.18</td>
<td>8.74±1.22</td>
<td>4.83±0.57</td>
<td>11.45±1.86</td>
</tr>
</tbody>
</table>

*p*<0.001, compared with PBS stimulation for PBMC.

### Table 2. Comparison of IL-10 levels in mononuclear cell culture supernatants with the bacteria antigen stimulation among psoriasis patients, chronic tonsillitis patients and control subjects (pg/ml)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>CWDB</th>
<th>Wild-type bacteria</th>
<th>PBS</th>
<th>PHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psoriasis patients</td>
<td>10</td>
<td>18.20±2.28</td>
<td>20.05±4.49</td>
<td>19.47±4.66</td>
<td>32.83±4.73</td>
</tr>
<tr>
<td>Chronic tonsillitis</td>
<td>10</td>
<td>18.56±2.36</td>
<td>19.91±2.30</td>
<td>18.04±2.83</td>
<td>31.63±5.01</td>
</tr>
<tr>
<td>Control subjects</td>
<td>10</td>
<td>18.77±2.33</td>
<td>18.66±3.73</td>
<td>19.27±5.05</td>
<td>27.98±4.59</td>
</tr>
</tbody>
</table>

*p*=0.329, no difference among psoriasis patients, chronic tonsillitis patients and control subjects.

### Table 3. Comparison of PBMC proliferations by bacterial antigens in psoriasis patients, chronic tonsillitis patients and control subjects (%)

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Remission (n (%))</th>
<th>Marked improvement (n (%))</th>
<th>Improvement (n (%))</th>
<th>No change (n (%))</th>
<th>Worse (n (%))</th>
<th>Effective rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psoriasis patients</td>
<td>10</td>
<td>11.1±0.05</td>
<td>1.10±0.06</td>
<td>0.76±0.10</td>
<td>1.63±0.23</td>
<td>1.81±0.09</td>
<td>84.4%</td>
</tr>
<tr>
<td>Chronic tonsillitis</td>
<td>10</td>
<td>11.1±0.05</td>
<td>1.12±0.07</td>
<td>0.69±0.10</td>
<td>1.81±0.11</td>
<td>1.81±0.09</td>
<td>84.4%</td>
</tr>
<tr>
<td>Control subjects</td>
<td>10</td>
<td>1.08±0.05</td>
<td>1.10±0.07</td>
<td>0.69±0.10</td>
<td>1.63±0.23</td>
<td>1.81±0.09</td>
<td>84.4%</td>
</tr>
</tbody>
</table>

*p*<0.001, compared with PBS.

### Table 4. The PGA of psoriasis patients at the end of the treatment

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Remission (n (%))</th>
<th>Marked improvement (n (%))</th>
<th>Improvement (n (%))</th>
<th>No change (n (%))</th>
<th>Worse (n (%))</th>
<th>Effective rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group A</td>
<td>45</td>
<td>15 (33.3)</td>
<td>9 (20.0)</td>
<td>14 (31.1)</td>
<td>7 (15.6)</td>
<td>0 (0)</td>
<td>84.4%</td>
</tr>
<tr>
<td>Group B</td>
<td>47</td>
<td>8 (17.0)</td>
<td>5 (10.6)</td>
<td>13 (27.7)</td>
<td>21 (44.7%)</td>
<td>0 (0)</td>
<td>55.3%</td>
</tr>
</tbody>
</table>

*p*=0.003, compared with the PGA of group B.

DISCUSSION

Over the past 10 years, most attention has been directed at cell-mediated immune mechanisms driving this increased epidermal proliferation of psoriasis. Many experimental studies provide evidence that psoriasis is largely a T-cell mediated disorder and the majority of the leukocytes in the dermis in psoriasis are CD4+ positive helper T-lymphocytes of the Th1 phenotype. The central role played by activated T-cells in animal model systems and the response of psoriasis to drugs that block T-cell activation have been demonstrated. As has already been noted, keratinocyte growth in psoriasis may be induced by a soluble factor produced by T-cells, probably IFN-γ. Various microorganisms are associated with the provocation and/or exacerbation of psoriasis, but their roles in the disease pathogenesis are unknown. Hence, there are conflicting views regarding the ethological factors of psoriasis. CWDB, pleomorphic bacterial forms, can be triggered by antibiotics, but it can also be a protective response to immune system. When the harsh environment is removed they are able to morph back into the active wild-type bacteria state and quickly propagate an active infection. In the CWDB state they are extremely hard to kill, and as they die they dump their toxic load into the cytoplasm of the phagocytes they have infected—the resulting endotoxin creating a Th1 cytokine cascade. Psoriasis may be considered to be a sterile antibacterial skin reaction mediated by T cells, during
CWDB infection, but the true relevance of CWDB to psoriasis is not analyze. Here, we research the relation between CWDB and psoriasis, and discover some evidences that psoriasis might be triggered by CWDB through introducing the activation of immune systems.

The investigations of our study to evaluate rates of carriage and acquisition of CWDB found about 75% psoriasis patients were infected with CWDB in the tonsil or pharynx compared with 23.5% chronic tonsillitis patients and 6.3% control subjects. The detection rate of CWDB carriage in psoriasis was remarkably lower after antibiotic therapy. Normal oropharyngeal flora consists of a variety of anaerobes and aerobes represented most prominently by *Viridans streptococci*. “Abnormal” oropharyngeal flora is opportunistic aerobic Gram-negative bacilli (AGNB), and considered potential pathogens.21 In the current study the rates for AGNB of CWDB were 79.3% in the pharynx of psoriasis patients. Overgrowth of CWDB, especially CWDB of AGNB, at the pharynx may pose a significant pathologic risk in psoriasis.

Although many isolates revenants were susceptible to β-lactam antibiotics in our study, we chose the antibiotics, except β-lactam antibiotics, to treat psoriasis patients according to the antibiotics sensitivity results because the hallmark of CWDB is the lack of a cell wall and lack of sensitivity to β-lactam antibiotics. Antibiotics therapy was appropriate for approximately 80% of cases with CWDB in the tonsil or pharynx. The fact is that antibiotic therapy, except β-lactam antibiotics, is appropriate for patient with psoriasis. In some studies it has been reported that intervention by antibiotics is not beneficial in psoriasis,7,8 it is main reason that CWDB are resistant to most antibiotics.

As has already been noted, the pathogenic T-cells in psoriasis are skewed towards the T1 phenotype, in that they secrete IFN-γ, IL-2, and TNF-α but not IL-4.11 In our study though IL-10 production was not altered, CWDB and wild-type bacteria did remarkably enhance the production of IFN-γ, *in vitro*. Meanwhile, proliferation of PMBC was activated by CWDB and wild-type bacteria. Therefore, CWDB and wild-type bacteria of AGNB may play an important role in the triggering of psoriasis, which up-regulates T-cell activation by activating some genes expression (such as IFN-γ).

The life cycle of CWDB is not well known. They may be triggered by antibiotics, lysozyme, and bile. When the inducing agent is removed, CWDB state may revert, or become stable. In our study an overwhelming body of evidence supports the concepts that CWDB, especially CWDB of AGNB, are proposed as a major etiological factor for psoriasis, which might induce psoriasis by the activation of immune systems through the exposed antigen. For chronic tonsillitis or pharyngitis of psoriasis patients, CWDB with wild-type bacteria, especially ANGB, can be carried continuously in the tonsil or pharynx. In the “bacterial storage pool”, CWDB can revert to wild-type bacteria and wild-type bacteria can become CWDB state when subject to the relevant stresses. Cross-reactive mononuclear cells in the tonsils or pharynx, especially T cells, proliferate and take up bacteria or antigens that were exposed after CWDB lost the membrane, and migrate to the skin. In the skin, an inherently altered response by psoriatic keratinocytes to cytokines released by these activated T cells, in particular IFN-γ, produces the characteristic features of hyperproliferation and incomplete differentiation of the epidermal layer. Antibiotics chosen according to CWDB and antibiotics susceptibility of wild-type bacteria revenant could be used to cut the pathogenic pathway in psoriasis.

Although it does not confirm that which microbial components of CWDB can stimulate immune systems in this disease, these observations should provide a stimulus for further research to identify and elucidate the role of this bacterial antigen in disease pathogenesis. In the future, increased knowledge in this area might lead to the identification of suitable targets for novel approaches to treating this common skin disease.

Based on the results of various studies carried out in relation to CWDB, it could be said that compelling evidence exists linking this microbe to psoriasis, provoking with recurrent acute tonsillitis or pharyngitis and exacerbating in the development of psoriasis. To prove the scientific logic of this possibility, and its benefit to patients clinical trials using anti-CWDB measures in psoriasis are required to be carried out in prospective longitudinal studies. Further studies are in progress to identify the antigen(s) involved, and to determine the potentially important role of these T cells in the immunological pathogenesis of psoriasis.

REFERENCES


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