Abstract

Background: Rheumatoid arthritis (RA) is a systemic autoimmune disorder causing synovitis of diarthroidal joints in which activated T-lymphocytes has a prominent position in RA pathology and may be important in prediction of disease outcome.

Objective: To evaluate cellular expression of certain activation marker on PBLs and their relevance to disease activity pattern of RA patients.

Patients and methods: this study included forty six RA patients, seven patients with Osteo-arthritis (OA) and 10 apparently healthy individuals. The collection of our baseline data based on routine laboratory and clinical assessment of disease activity Score (DAS). Blood sample was taken from each subject in all groups, at the time of attendance. Lymphocytes were separated; slides were prepared fixed on charged slides, foiled, and kept at -20ºC until assayed. CD3 and CD54 expression was detected using Immunocytochemistry staining, while CD71 was detected using direct immunofluorescence staining.

Results: The results of CD3 and CD54 revealed a statistically higher percentage of expression in rheumatoid arthritis patients when compared with that of the apparently healthy and OA control groups. The CD71 showed statistically significant higher expression in minimum disease activity group without any correlation with clinical and laboratory disease activity indices.

Conclusions: we provide further evidence of a T-cell differentiation defect in RA, which could explain some of the well-characterized immunologic features of the disease but it is not related to the disease activity state.

Keywords: Rheumatoid arthritis, disease activity, lymphocytes activation, immunocytochemistry, immunofluorescence and CD marker.

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Introduction

Rheumatoid arthritis (RA) is a quintessential autoimmune disease with a growing number of cells, mediators, and pathways implicated in this tissue-injurious inflammation (1). It’s strongly suggested that it was driven by specific T-cell-mediated cellular immunity against self-antigens and the T-cell-mediated cellular immunity was proposed to be involved in RA pathology (2, 3).

Intercellular adhesion molecule-1 (ICAM-1) is a cell surface molecule that have been expressed upon lymphocyte activation, ICAM-1 gene expression on T cells regulated by Phosphotyrosyl Phosphatase Activity (4), and only mitogens or specific cytokines increase its expression on T lymphocytes (5-7).

The membrane glycoprotein transferrin receptor (TfR, CD71), In addition to being an iron transporter, it has been shown to play a role in T cell activation. Stimulation of the TfR with specific Abs results in T cell proliferation, IL-2 secretion, and protein kinase C activation. It appears to play as a costimulatory role in T cell activation (8,9).

Those markers were reported to play an important role not only in T cell recruitment, but also in T cell activation, proliferation, and in the development of specific immune responses. Furthermore, circulating
leukocytes provide an important source for biomarker discovery for RA. In contrast to target tissue biopsy based approaches, which are often limited by restricted access to target tissues, profiling peripheral blood cells has emerged as an attractive biomarker discovery strategy [10, 11].

This study aimed to investigate the expression of immune alteration phenotype on PBL, like the expression of CD54 and CD71 and to correlate the results of PBL-alteration phenotype expression with different disease activity patterns.

**Materials and methods**

**Subjects:**

The present study groups consisted of 46 Iraqi patients with RA fulfilled the ACR classification criteria [12]. They were recruited from the outpatient clinic at the Department of Rheumatology and Rehabilitation, Al-Kadhumyia Teaching Hospital in Baghdad. Also 7 age-and sex-matched osteo-arthritis patients and 10 apparently healthy controls were enrolled in the study. These controls were healthy blood donors.

The scoring system of the present disease activity was done according to modified DAS28-3. It combines of both clinical and laboratory parameters. The clinical examination of joint swelling and tenderness was performed for 28 joints (include the same joints: shoulders, elbows, wrists, metacarpophalangeal joints, proximal interphalangeal joints and the knees [13]).

General laboratory and immunolaboratory assessments included erythrocyte sedimentation rate, C-reactive protein, and RF. Clinical and laboratory characteristics of the patient included in the study are summarized in Table 1.

**Blood samples:**

A Blood sample (5 ml venous blood) was aspirated from a suitable vein from all patients and unaffected controls. Blood was collected in pyrogen-free silicone-coated tubes with heparin. The blood samples were used for lymphocyte separation according to Isopaque-ficol technique (originally described by Boyum in 1968) [14].

Heparinised peripheral blood was diluted 1/1 with phosphate buffered saline (PBS), and mononuclear cells were isolated by ficoll density gradient centrifugation at 2000 rpm for 20 minutes. Mononuclear cells were washed three times with PBS for 5 minutes, resuspended at 2x10^6cells/ml, and fixed on poly-L-lysine-coated glass slides, wrapped, and kept at -20°C until assayed.

**Immunocytochemistry staining method:**

Briefly, the precoated charged slides were removed from freezer, allowed to reach room temperature, unwrapped and then dipping the slides into PBS-filled jar for about 5 minutes and slides were placed on a flat level surface, then endogenous peroxidase was quenched by initial incubation of the smears by enough drops of Peroxidase block for 5 minutes at room temperature then rinse with PBS from a washing bottle. The slides then placed in PBS wash bath for 2 minutes and excess buffer were taped and wiped around smears. Then, enough power block reagent (1/10 diluted in PBS) were applied for 5 minutes and excess blocking reagent was taped but not washed to avoid non-specific binding of antibodies. Then, the coated lymphocytes were covered by 20 μl of 1/30 diluted mouse monoclonal Ab (primary Ab) specific human CD-marker (CD3 and CD54). Slides then incubated at 37°C for 1hr, and then unreacted monoclonal Ab was removed by three cycle for 2 minutes of washing with PBS. Then they were washed and wiped around the smear.
After that enough solution of biotinylated secondary antibody (anti-mouse Ab) was applied to cover each smear, distributed evenly over the precoated slides then placed in humid chamber for 1 hour at 37°C and washed in buffer and bathed in PBS for 5 minutes then wiped around smear. Enough solution of streptavidin conjugated peroxidase was applied to cover the smear and slides were placed in humid chamber for 1 hour at 37°C. Then they were washed in buffer, bathed in PBS for 5 minutes and wiped around the wells. Then enough drops of freshly prepared DAB working solution were applied to cover the section at room temperature for 10 minutes or until the color was observed and the reaction was terminated by rinsing gently with distilled water from a washing bottle. Slides then were placed in bath of hematoxyline for 30 seconds at room temperature. Slides were rinsed gently with distilled water from a washing bottle then and under gently running tap water for 5 minutes. A drop of mounting medium (DPX) was placed onto the wet smear and the spot were quickly covered with a cover slip and left to dry.

The slides were examined under 40X-magnification power of light microscope (ZEISS). The dark brown (homogenous or membranous) staining identified positive labeled cells as in figure (3).

**Direct immunoflourescence staining method:**

The slides were prepared as described previously. Then, the precoated slides with lymphocytes were removed from freezer, allowed to reach room temperature, unwrapped and washed with PBS by dipping the slides into PBS- containing jar for about 5 minutes at room temperature. They were laied flat, smear-side up, in humidity chamber, then 20μl of 1/30 diluted fluorochrom (FITC) conjugated monoclonal antibodies were added to each smear, cover chamber and slide were left undisturbed in incubator at 37°C for 50 minutes. Slides then transferred to staining jar filled with PBS at room temperature and PBS replaced twice at 5 minutes intervals, then drained and blotted gently.

Two drops of mounting media [(nine parts glycerol to one part of 0.2M carbonate buffer, pH=9) to enhance fluorescence and retard fading on exposure to UV-light (16) were placed on each smear of slides. Then cover slips were lowered into place slowly to avoid bubbles; cover slips may be sealed around edges with clear nail polish. Slides were examined then with fluorescence microscope at 490 nm; positive cells give green-apple when stained with FITC-labeled antibodies and exposed to UV light.

**Statistical analysis**

The percentage of each of the tested marker expression on PBLs was calculated by a simple calibration of percentage of reactivity as in following formula:

\[
\text{Percentage of expression} = \left( \frac{\text{No. of positive cells}}{\text{total No. of cells}} \right) \times 100\%.
\]

Statistical differences in measured values were analysed using independent sample-test. P-values <0.05 were considered statistically significant.

**Results**

**T cell activation phenotype:**

Among the studied markers, there is significantly increased percentage of T-cell population in the peripheral circulation of RA patients compared with the percentage found in patients control and healthy individuals. Also, significantly elevated percentage of expression of functional activation antigen (CD54) and early activation antigen (CD71) were found in (table 2) (figure 1, 2).

Patients were divided (based on
modified DAS28-3) in to two groups: high disease activity group (37 patients) and minimum disease activity group (9 patients). There was no statistical significant difference in the mean percentage of cells that express CD3. While, CD54 showed no statistical significant difference with lower percentage in active disease group and CD71 showed statistically significant difference with higher expression in minimum disease activity group (table 3).

**Lack of influence of PBLs alteration phenotype with clinical and laboratory indices:**
Our results showed no statistical correlation between studied markers and different clinical and laboratory parameters for disease activity (Table 4).

![Figure 1: percentage of expression of PBLs markers in different study groups.](image1)

![Figure 2: Percentage of expression of PBLs markers in RA patients subgroups.](image2)
Table 1: patients and control characteristics. data are presented as means (SE).

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>Osteo-arthritis group</th>
<th>RA patients</th>
<th>RA patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>RA patients</td>
<td>Minimum disease activity group</td>
</tr>
<tr>
<td>Women/men</td>
<td>9/1</td>
<td>6/1</td>
<td>42/4</td>
<td>34/3</td>
</tr>
<tr>
<td>Age/years</td>
<td>48.6(10)</td>
<td>45.76 (7.6)</td>
<td>47.67(12.09)</td>
<td>48.06(11.96)</td>
</tr>
<tr>
<td>Disease duration (months)</td>
<td>----</td>
<td></td>
<td>88.61(72.88)</td>
<td>92.34(68.28)</td>
</tr>
<tr>
<td>ESR (mm/1^h)</td>
<td>12.50(3.31)</td>
<td>45.6 (12.5)</td>
<td>67.43(20.26)</td>
<td>70.94(19.54)</td>
</tr>
<tr>
<td>CRP (mg/l)</td>
<td>10.20(15.24)</td>
<td>22.4 (16.5)</td>
<td>43.956(55.078)</td>
<td>49.78(59.53)</td>
</tr>
<tr>
<td>Tender joints</td>
<td>----</td>
<td></td>
<td>10.58(5.42)</td>
<td>12.54(4.62)</td>
</tr>
<tr>
<td>Swollen joints</td>
<td>----</td>
<td></td>
<td>7.35(4.52)</td>
<td>8.63(4.36)</td>
</tr>
<tr>
<td>DAS-28(3)</td>
<td>----</td>
<td></td>
<td>5.77(0.83)</td>
<td>6.11(0.63)</td>
</tr>
<tr>
<td>RF sero-positive (%</td>
<td>2(21.4%)</td>
<td>1 (16.6%)</td>
<td>34 (73.9%)</td>
<td>27(72.9%)</td>
</tr>
<tr>
<td>Duration of morning stiffness (minutes)</td>
<td>----</td>
<td></td>
<td>76.41(41.30)</td>
<td>84(41.72)</td>
</tr>
</tbody>
</table>

ESR=erythrocytes sedimentation rate, CRP= C reactive protein, DAS= disease activity score, RF=rheumatoid factor.

Table 2: descriptive statistics (mean±S.E.) of PBLs markers in different study groups, comparison was done using ANOVA test.

<table>
<thead>
<tr>
<th></th>
<th>healthy group</th>
<th>OA group</th>
<th>RA group</th>
<th>ANOVA test</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>72.042±1.52</td>
<td>74.3±0.99</td>
<td>79.213±1.4</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>CD54</td>
<td>3.7±2</td>
<td>9.142±4.59</td>
<td>44.554±13</td>
<td>&lt;0.001 **</td>
</tr>
<tr>
<td>CD71</td>
<td>2.3±1.82</td>
<td>4.571±1.98</td>
<td>25.534±9.59</td>
<td>&lt;0.001 **</td>
</tr>
</tbody>
</table>

**: highly statistical significant difference at the level of p<0.001.

Table 3: Descriptive statistics (mean±S.E.) of PBLs markers in different RA subgroups, comparison was done using independent sample t-test.

<table>
<thead>
<tr>
<th></th>
<th>Active</th>
<th>Moderate</th>
<th>Sig. 2 tailed</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>79.242±1.39</td>
<td>79.061±1.66</td>
<td>0.761 NS</td>
</tr>
<tr>
<td>CD54</td>
<td>43.67±12.36</td>
<td>48.85±15.12</td>
<td>0.336 NS</td>
</tr>
<tr>
<td>CD71</td>
<td>29.198±9.08</td>
<td>32.885±9.7</td>
<td>0.039*</td>
</tr>
</tbody>
</table>

NS: no statistical significant difference.
*: statistical significant difference at the level of p<0.05.
Table 4: Correlation matrix between studied PBLs markers and disease activity parameters.

<table>
<thead>
<tr>
<th>Pearson Correlations</th>
<th>CD 3</th>
<th>CD 54</th>
<th>CD 71</th>
</tr>
</thead>
<tbody>
<tr>
<td>TJC</td>
<td>0.028</td>
<td>-0.079</td>
<td>-0.233</td>
</tr>
<tr>
<td>SJC</td>
<td>-0.062</td>
<td>0.116</td>
<td>-0.022</td>
</tr>
<tr>
<td>ESR</td>
<td>-0.183</td>
<td>0.008</td>
<td>0.107</td>
</tr>
<tr>
<td>RF</td>
<td>-0.022</td>
<td>0.035</td>
<td>-0.023</td>
</tr>
<tr>
<td>CRP</td>
<td>0.016</td>
<td>0.092</td>
<td>0.072</td>
</tr>
<tr>
<td>DAS-28(3)</td>
<td>-0.065</td>
<td>-0.050</td>
<td>-0.149</td>
</tr>
</tbody>
</table>

Discussion

Our results has made it obvious that the strong up-regulation of both CD54 (functional activation antigen) and CD71 (early activation antigen) give a strong evidence that PBLs were within a state of immune dysregulation. That comes together with a recent study done by Poriadin and his co workers in 2006; who demonstrated that the increased expression of activation induced antigens (including CD54 and CD71) on the PBL from patients with various types of inflammatory disorders. It is abnormal regulation of activation processes of lymphocytes in allergic and autoimmune disease consistence in the absence of lymphocyte activation inhibition (17).

Of note, the persistent PBL expression of activation markers occurs due to impaired activation induced cell death (AICD) with failure in the late cell cycle stages. Tang and coworkers in 2004 tried to demonstrate this abnormality and found that there was a soluble survival signal present in RA patient’s serum as well as, synovial fluid produced by CD14+ve cell and its secreted form (18). In addition, effector and memory lymphocytes were also reported to accumulate in the peripheral blood and synovium of RA patients (19, 20). Although, the exact mechanism(s) for accumulation of those atypical T lymphocytes are still incompletely understood; there may be three possibilities for accumulation and expansion of autoreactive lymphocytes in RA patients: first is a continual input of autoreactive lymphocytes into the peripheral lymphocyte pool, certain genetic backgrounds may predispose an individual to accumulate autoreactive T cells in vivo (21,22), the second is a failure to suppress autoreactive lymphocytes via anergy (23,24), the third possibility is a failure to remove autoreactive lymphocytes from the peripheral lymphocyte pool by apoptosis (25). That may be due to impaired balance between pro-apoptotic (Bax) and anti-apoptotic (Bcl2 and Bclxl) proteins which well known regulator in programmed cell death. Furthermore, the autoreactive lymphocytes were switched from apoptosis-sensitive to apoptosis-resistance state (26, 27).

Our results failed to find a correlation between the expression of CD54 and CD71 with clinical and other laboratory indices. Such a results are in agreement with previous study done by Buckley, 2003 who suggested that the chronic inflammation which occurs may be due to impaired dynamic of inflammatory infiltrate that postulate rheumatoid joint as a “foster home” for PBL in which wrong cells...
(PBL) accumulate in the wrong place (joints) at the wrong time (during the resolution of inflammation) leading to improper retention and survival (28).

In summary, we have confirmed and illuminated some of the T-cell differentiation defects in RA. Our data reinforce the importance of early and aggressive therapy for RA. Although T-cell abnormalities may predate clinical signs and symptoms, they appear to be perpetuated by inflammation. Therefore, the control of inflammation, particularly through the use of cytokine blockade, should minimize dysregulation of proliferation.

References
16. Boyum A. lymphocyte separation, Scan J Clin Lab Invest.1968; 21(97).


