Inflammatory Cytokine Secretion Status of Bone Marrow Cells and Clinical Significance in Immune-related Hematocytopenia

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Objective To observe the expression of inflammatory molecules in bone marrow immune cells of patients with immune-related hematocytopenia (IRH), and to investigate the immune mechanism and clinical significance of the disease.

Methods Total of 36 IRH patients were selected as observation group and 30 healthy people were taken as control group. Serum cytokines levels, activity of immunocytes and expression of HLA-DR were detected. Immune fluorescence was applied to observe the expression state of immunologic molecules and cytokines in IRH patients.

Results Serum cytokines were elevated in various degrees in observation group. Compared with the control group, the cytokines levels were significantly higher ($P < 0.05$). After treatment with immunosuppressive drugs, the serum levels of cytokines in observation group reduced to a level close to the control group. HLA-DR were upregulated in activated tissue basophils, eosinophils, dendritic cells (DC) and macrophages of bone marrow in IRH patients, and POX activity in these immunocytes of IRH was higher than that of the control group. Immune molecules were highly expressed in eosinophils, DC and macrophages.

Conclusions It is demonstrated that antibodies or self-reactive lymphocytes were produced in IRH marrow, which would cause lesions of hemocytes, and lead to pathological process finally. Structure of hematopoietic cells mutated and these cells might be acted as target cells of immunocytes in the pathological process. Immunocytes could secrete inflammatory factors and lead to immunologic injury of hemocyte.

Key words: Immune-related hematocytopenia; Basophils; Eosinophils; Anti-human IgG immunoglobulin; IL-17AL; IL-17RA; Immune molecules

With the incidence of rheumatic disease increasing and the improvement of diagnostic methods, more and more patients with rheumatic-immune-related hematocytopenia (RIRH) were diagnosed. RIRH is considered to be a severe disease, which is associated with many factors. Extensive studies have demonstrated that RIRH patients always had hypocytosis or pancytopenia in addition to some autoimmune diseases.

RIRH is considered to be a multisystem disorder and the clinical situation is always complicated. Two

or three series of haemocytes decreased abnormally, bone marrow hyperplasia is always active or normal, and immunocytes in bone marrow are activated and a great quantity of inflammatory cytokines are secreted, and myeloid element are damaged due to pathological immune response, and glucocorticoid treatment has been reported to be effective in some cases. Many factors may play roles in this disease, and autoimmune disorder may play a critical role in the process.

There are limited studies on the cellular and humoral immune responses in RIRH. In this study, we analyzed the secretion of inflammatory cytokines in serum and bone marrow, and we presented preliminary evidence of immunological effect in

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IRH and hope to provide clues for pathogenesis and therapy of RIRH.

PATIENTS AND METHODS

Patients

Total of 36 patients (from the 148th Hospital of PLA, Zibo, Shandong Province, China) who were diagnosed as RIRH were enrolled in our study from 2008 to 2011, including 16 males and 20 females, aging from 36 to 72 years old. The study protocol was approved by the Hospital's Ethical Committee. The diagnostic standard was coincided with literature protocol and confirmed by laboratory demonstration. Totally, 21 cases were diagnosed as pancytopenia and other cases were diagnosed as hypocytosis involving two series of haemocytes. Blood analysis: white blood cell (WBC) counts $(0.7 - 3.2) \times 10^9/L$, hemoglobin (HB) concentration $(35 - 102) g/L$, blood platelets (PLT) counts $(30 - 238) \times 10^9/L$. And 7 cases were diagnosed as Sjögren's syndrome (SS); 15 cases as rheumatoid arthritis (RA), and rheumatoid factor (RF) of 8 rheumatic heart disease (RHD) patients increased abnormally; 4 cases were with systemic lupus erythematosus; 2 cases were with mixed connective tissue disease (MCTD); 9 patients with $WBC < 2.0 \times 10^9/L$ were accompanied with pulmonary infection and urinary tract infection, the erythrocyte sedimentation rate (ESR) and IgG level of these 9 patients also increased abnormally; 11 patients were administered with intermittent treatment of glucocorticoids. HLA-B27 level of 16 cases (44.44%) increased (ankylosing spondylitis, AS were excluded with image examination); antinuclear antibody of 9 patients were positive. And 30 healthy natives from medical examination center of the hospital were taken as the control group (15 males and 15 females aging 68 - 76 years old).

Reagents and equipments

The ELISA kits for human cytokines were purchased from RD Company, goat anti-human HLA-DR monoclonal antibody (Mab) from Beyotime Company, ALP immunocytochemistry kit from Beyotime Company. Other antibodies including goat anti-human IgG antibody, Cy3-labeled donkey anti-goat IgG fluorescence secondary antibody, mouse anti-human Fcγ II R Mab, FITC-labeled goat anti-mouse IgG fluorescence secondary antibody, PE-labeled mouse anti-human mannose receptor (MR) (CD206),

AF488-labeled mouse anti-human IL-12, FITC-labeled mouse anti-human IL-17A and PE-labeled mouse anti-human IL-17Ra Mab were purchased from Santa Cruz Company. Olympus BX51 microscope assembled with reflected fluorescence system was used for microscopic analysis.

Determination of cytokines levels

About 3 - 5 ml peripheral blood were collected into sterile tubes from patients at pre-treatment and post-treatment stages, also for members in the control group, then sera specimens were separated and collected, all samples were stored at $-80^\circ C$. Both pre-therapy and post-therapy sera of clinically cured patients were collected. Enzyme linked immunosorbent assay (ELISA) was applied to quantitatively determine cytokine levels in serum such as IL-5, IL-6, IL-10, IL-12, IL-17 and IFN-γ for both pre-therapy and post-therapy. Triplicates were set up for each cytokine per sample, the operative procedure were strictly performed according to the instruction manuals provided with the ELISA kit. The A value were detected at 450 nm using a detector (Thermo Scientific, Multiskan FC, USA) and the average value of triplicates were calculated as final true value for each cytokine, then standard curve were drawn and fitted with Expert Curve software according to the data of standards, and serum cytokine levels were converted according to the standard curve.

Immunocytochemistry and immunofluorescence staining

Bone marrow slides were prepared when patients were treated with bone marrow aspiration before and after clinical therapy. Wright's staining was performed to observe the hematopoietic cell proliferation state as well as the active state and proportion of immunocyte. BENZ staining was used to observe the peroxidase (POX) of immunocyte. Immunocytochemistry staining was performed to analyze the expressive state of HLA-DR antigen located on the membrane. Immunofluorescence method was applied to observe the excretive and expressive levels of immunomolecules including anti-human IgG antibody, Fcγ II R, MR, IL-12, IL-17a and IL-17Ra induced by marrow immunocyte. In brief, the bone marrow tissue slides of iron deficiency anemia (IDA) patients were selected as comparison (marrow slides of healthy people were not available). The immunological state of myeloid elements, including the amount and distribution of mononuclear macrophage and other

immunocyte as well as the changes of enzyme activity reflected by immunohistochemical staining color, were observed to evaluate the immunocyte activity in bone marrow.

Therapeutic regimens

Glucocorticoids: Prednisone for oral application and hexadecadrol or methylprednisolone for intravenous drop was selected to induce apoptosis of lymphocytes and depression of immunocytes according to the pathogenetic condition of patients at a dose of 1 - 1.5 mg·kg⁻¹·d⁻¹, and after hemogram recovering to normal, the medicine dosage was decreased progressively. **Anti-infective therapy with antibiotics:** according to the results of bacterial culture and susceptibility detection, suitable antibiotics associated antiviral drug were selected for patients with infection, fever and low level of WBC.

Cyclosporin A (CsA): In addition to anti-infective therapy associated with glucocorticoids treatment, patients with high level of HLA-B27 and elevation of ESR (HLA-B27⁺ESR⁺) were also given CsA at a dosage of 5 mg·kg⁻¹·d⁻¹ for oral use to suppress T helper cell activity.

Improvement on pathological change of vasculitides: patients with PLT > 60×10⁹/L received treatment of intravenous drop to improve the inflammation of blood vessel. Symptomatic treatment and supportive treatment were also performed to promote the rehabilitation of patients.

Statistical analysis

Data analysis was described by mean ± SD or medians. Variability between the two groups was analyzed by *t* test with 95% confidence interval, percentage data was analyzed with χ^2 test. Correlations were evaluated through Spearman's correlation test. SPSS 13.0 software was applied for the statistical analysis. *P* < 0.05 was considered to be statistically significant.

RESULTS

Analysis of serum cytokines

Before the treatment, serum levels of cytokines IL-5, IL-6, IL-10, IL-12, IL-17 and IFN- γ were (126.04 ± 7.66) pg/ml, (136.23 ± 16.0) pg/ml, (316.23 ± 25.163) pg/ml, (328.43 ± 27.01) pg/ml, (48.17 ± 4.22) pg/ml and (40.28 ± 3.69) pg/ml, respectively, with significant deviation, all of *P* values < 0.05. For the observation group, after four weeks therapy when the hemogram recovered to normal, serum cytokine levels of the above indexes decreased to (38.21 ± 5.14) pg/ml, (88.56 ± 10.13) pg/ml, (188.13 ± 12.51) pg/ml, (96.01 ± 6.76) pg/ml, (26.01 ± 4.23) pg/ml and (7.13 ± 2.55) pg/ml, respectively, with significant decrease compared with those of pretherapy (*P* < 0.05) (Table 1). Besides, the rates of elevated IL-5 and IL-17 for patients before treatment, were 100% (36/36), while 97.22% (35/36) for elevated IL-12, which were significantly higher than the control group, with *P* values shown in Table 1. Similarly, compared with healthy controls, the elevated levels of IL-10 (24/36, 66.67%) and IFN- γ (26/36, 69.44%) were significantly higher (*P* < 0.05).

POX and HLA-DR expression of immunocytes

The amount of tissue basophil cell in bone marrow of RIRH patients with SS, patients with RA and RF⁺ patients with RHD, as well as patients with abnormally increase of serum IgG and ESR (IgG⁺ESR⁺) increased obviously, the increase percentage of tissue basophil was (3.20 ± 0.08)% in differential blood count, a predominant increase compared with healthy controls (0.6 ± 0.05)%, *P* < 0.05. From the bone marrow slides, activated tissue basophils were found to adhere and capture mutated haemocyte (Figure 1A), even to attack marrow haemocytes by releasing basophilic stippling. Correlation test also showed that the increase of tissue basophils was correlated to IL-10, with the correlation coefficient *r* = 0.885. EOS amount of observation group elevated and reached (7.4 ± 3.1)% in differential blood count vs. (2.4 ± 1.6)% of the healthy control (*P* < 0.05). POX in specific granules of those activated EOS was positive, and mutated hematopoietic cells could be found to

Table 1. Levels of serum cytokines (pg/ml)

	IRHS (n = 36, $\bar{x} \pm s$)		Controls (n = 30, $\bar{x} \pm s$)	<i>P</i> value Pre-therapy vs. controls
	Pre-therapy	Post-therapy		
IL-5	126.04 ± 7.66	38.21 ± 5.14	33.16 ± 2.17	< 0.0001
IL-6	136.23 ± 16.00	88.56 ± 10.13	90.98 ± 12.32	< 0.0100
IL-10	316.23 ± 25.16	188.13 ± 12.51	176.25 ± 10.69	< 0.0500
IL-12	328.43 ± 27.01	96.01 ± 6.76	94.05 ± 3.26	< 0.0001
IL-17	48.17 ± 4.22	26.01 ± 4.23	20.03 ± 1.14	< 0.0001
IFN- γ	40.28 ± 3.69	7.13 ± 2.55	8.25 ± 2.01	< 0.0010

Figure 1. Strengthened POX activity and highly expression of HLA-DR in activated immunologic cells in bone marrow.

Notes: A. An activated tissue basophil; B. An neutrophil captured by an paramorph M ϕ (shown by arrow); C. An activated paramorph EOS; D. A pathological neutrophil with degeneration of nucleus (shown by arrow); E. POX activity was highly expressed by EOS, and a pathological neutrophil with poor plus activity of POX was attacked by the EOS (shown by arrow); F. Activated and paramorph EOS expressed POX at very high level; G. Plasmocyte was in paramorphia after glucocorticoids treatment; H. An activated tissue basophil cell showed highly positive expression of HLA-DR; I. An enormous and activated M ϕ expressed HLA-DR at high level and captured a great quantity of haemocyte; (A, B, C, D, E, F, G, H were at a magnification of 1000 $\times$, I was at a magnification of 400 $\times$)

be adhered, captured or even swallowed by these activated EOS. Also, POX of neutrophils attacked by EOS was weakened obviously (Figure 1C-F). Hematopoietic cells attacked by dendritic cells (DC) and activated lymphocytes could be observed easily. Inflammatory cells were found to be recruited by those activated M ϕ (Figure 1B) in the bone marrow of patients with infection. Diastrophic Mo-M ϕ with positive POX activity could be easily discriminated in bone marrow of patients with intermittent treatment of glucocorticoids. HLA-DR expression of immunocytes in marrow could be analyzed by immunocytochemistry, which were strongly expressed and significantly elevated of activated DC, M ϕ , EOS and tissue basophils, but for most patients treated with intermittent glucocorticoids, DC, M ϕ and EOS showed negative expression of HLA-DR (data not shown). Plasmocytes were topically hyperplastic, with suppressed secretion. Cytoryctes could be observed in marrow of patients with positive antinuclear antibody (Figure 1D).

The expression and distribution of anti-human IgG antibody in bone marrow

In bone marrow of IRH patients accompanied with Sjögren's syndrome (SS), rheumatoid arthritis (RA) and rheumatic heart disease (RHD), IF of anti-human IgG antibody was girdle-shaped and strongly positive in/on the cytoplasm and cell membrane of immature erythrocyte and immature granulocyte. But in bone marrow of SLE patients, IF of anti-human IgG antibody was negative for immature erythrocyte and immature granulocyte, and M ϕ could be found swallowing anti-human IgG grains in marrow (Figure 2A-C). The granules of EOS and tissue basophil showed positive anti-human IgG antibody (data not shown). Anti-human IgG antibody grains were found to be adhered or captured in the intracytoplasm of activated M ϕ . IF staining of anti-human IgG antibody was negative in the marrow of healthy controls.

Expression of inflammatory molecules

Fc γ II R (Figure 2D-F) and adhesion molecule ICAM-1 (Figure 3C) were strongly positive in activated EOS, M ϕ and DC, but Fc γ II R was weakly expressed in tissue basophils. Activated EOS, M ϕ and DC could express IL-12 in secretor state (Figure 3B), but after therapy of immunodepressants,

Figure 2. Mφ would capture target haemocytes which express anti-human IgG and Fcγ II R on membrane in bone marrow of the patients group (1000 ×).

Notes: A-C: Activated Mφ were trying to capture a large anti-human IgG molecule and target pathological hematocyte; D-F: Fcγ II R receptor was expressed on the surface of Mφ.

Figure 3. Inflammatory cytokines were secreted in activated Mφ and MR, mediating inflammatory reaction and destroying hematopoietic cells indirectly in bone marrow (1000 ×).

Notes: A. Pathological hemocyte with lesions were captured and swallowed by activated Mφ (shown by arrow); B. IL-12 expressed by activated Mφ; C. ICAM-1 expressed by activated Mφ; D. In bone marrow of patients with activated humoral immunity, Mφ expressed IL-17A very weak molecule; E. In bone marrow of patients with activated cell immunity, Mφ expressed IL-17A molecule at a high level; F. Mφ highly expressed MR receptor to swallow a large of pathological hemocyte in bone marrow of patients with infection.

IL-12 secretion was suppressed obviously. Small amount of lymphocytes was found to be IL-17A⁺ in bone marrow of HLA-B27 heightened patients. But activated EOS and Mφ with positive IL-17A could be found easily (Figure 3D), and a large amount of IL-17A positive grains (Figure 3E) could be found in cytoplasm of Mφ of patients with infection, but secretion by Mφ was suppressed after immunodepressant treatment. IL-17RA molecule was widely expressed in many kinds of myeloid cells in bone marrow of patients with infection. MR (Figure

3F) was observed to be expressed in activated DC, Mφ and EOS. In comparison group of IDA, some marrow hematopoietic cells could express IL-17RA weakly (datas not shown), EOS and Mφ could secrete IL-12 and IL-17A, and weak Fcγ II R, ICAM-1, MR expression, which were not found to be expressed in DC.

The efficacy of immunodepressive therapy

After two weeks of glucocorticoids therapy associated with anti-infection drug treatment, ESR and IgG levels of most RIRH patients decreased obviously,

and hemogram was normal gradually. But for RIRH patients with pancytopenia and HLA-B27⁺, restoration process of hemogram were very slow and the activity of EOS was not improved significantly. After medicine therapy associated with CsA, hemogram of patient started to recover, and the POX activity of EOS was weak in marrow, and the apoptosis of Mφ was also observed, parts of DC and Mφ showed negative expression of HLA-DR (data not shown).

DISCUSSION

It is presumed that rheumatic immune disease is related to the disorder of autoimmunity. Many risks such as physical, chemical and biological factors could induce immunologic response of immune system against self component. Due to those autoantibody and autoreactive T lymphocytes produced by self immune system, adaptive immunity response against self normal antigens is induced in vivo. Because the autoimmune response is chronic and this would lead to the recurrent attacks of the disease. Furthermore, due to the molecular mimicry of autoantibody and the extension of abnormal autoantigen epitope, the pathological changes of hematopoietic system would occur finally. The antigen located on the cellular membrane of haemocytes might be modified, and then autohemoantibody and specific cytotoxic lymphocyte (CTL) would be produced, all the above would lead to hypersensitivity and patho-reaction and finally IRH would occur.

The hematologic examination of most RIRH patients revealed abnormally decreased haemocytes, active bone marrow hyperplasia, activated immunocytes in marrow and haemocytes swallowed by Mφ. Hematopoietic cell islands could be easily found in marrow slides of patients with ESR and increased IgG (ESR⁺IgG⁺). As karyocytes expressing MHC- I and immature haemocytes are the main target cells, IgG was positive on cellular membrane of immature erythrocytes and granulocytes by IF staining. Though destruction of mature erythrocyte could be found, Coombs' test of peripheral blood is rarely positive. Because of the protopathy, autoantibodies such as antinuclear antibody, antimitochondrial antibody and anti-human IgG antibody could be found in sera, and RF increases abnormally in sera of some non-RA patients.

Tissue basophils, also named as mastocytes, are considered to be derived from bone marrow stem

cells, which can secrete IL-1, IL-3, IL-4, IL-5, IL-6, IL-10 as well as chemotatic factors and other immune factors. Recently it is reported that mastocytes participated in the pathologic changes of autoimmune disease (AID), which might be related with the process of RA. In present study, it is found that the amount of tissue basophils increased abnormally in the marrow of RIRH patients with SS, RA and RHD accompanying increased RF as well as patients with IgG and ESR abnormally heightened, and the result was correlated with the level of serum IL-10. Those activated tissue basophils could be observed to attempt to adhere and capture hematopoietic cells, or even to release basophilic stippling. Anti-human IgG antibody of basophilic grains was positive, but IF of Fcγ II R were weakly positive, which indicated that tissue basophils might play an important role in the activation of humoral immunity and also mediate the damage of hematopoietic cells in the process of RIRH.

As both DC and Mφ are important in inherent immunity and adaptive immunity response, in we found that activated DC and Mφ could express various immuno-molecules, and these effector cells could adhere and capture hematopoietic cells through adhesion molecule ICAM-1 or release high concentration of POX and other enzymes to destroy haemocyte in marrow, they also could induce the cell conjugation by Fcγ II R expressed on the membrane and initiate antibody-dependent cell mediated cytotoxicity (ADCC) to kill target haemocytes, or swallow mutated haemocytes through MR receptor. HLA-DR was also found to be expressed in DC and Mφ, with the assistance of antigen presentation induced by HLA-B27, adaptive immunity response was activated and participated in the pathologic change, meanwhile DC and Mφ would secrete IL-12 and lead to the activation of cytoimmunity, then CTL induced by Th1 would also destroy those mutated haemocytes.

IL-17 is an important inflammation mediator secreted by Th17, which can mediate inflammatory reaction as well as tumor immunity, and is related to the process and development of AID. IL-17A is the most important proinflammatory factor of IL-17 family. IL-17A receptor (IL-17AR) is quite widely distributed, especially in hematopoietic tissues. The binding of IL-17A and IL-17AR could induce target cells to excrete IL-1β, IL-6, IL-8, iNOS, LIF, ICAM-1, GM-CSF, PGE2 and other inflammatory

factors, which would also promote the differentiation and maturity of DC and to regulate the process of hematopoiesis, inflammation and immunity. IL-17 can also stimulate immunocytes to excrete vary chemotatic factors, and induce the production of prostaglandin-E2 (PGE2) through cyclooxygenase-2 (COX-2).

IL-17 enlists granulocytes, lymphocytes and M ϕ to participate in the inflammatory reaction and lead to the destruction of modified haemocytes.¹⁻⁴ Our preliminary evidence suggested that DC, M ϕ and EOS can secrete IL-17A, which was related with the increase of IL-17 in serum. In addition, we demonstrated that the IL-17A secretion of DC, M ϕ and EOS was suppressed obviously after hemogram of peripheral blood was normal after immunodepressant treatment, which showed that IL-17A plays an important role in the process of RIRH. In comparison group of IDA, only a small quantity of haemocytes expressed weak IL-17A, while in the marrow of patients with infection, IL-17A expression was strongly and widely positive, which indicated that infection might aggravate the inflammatory damage to myeloid elements induced by IL-17A. So seeking for the inhibitor of IL-17A and trying to interrupt inflammatory damage induced by IL-17A may be sensitive and clinically meaningful.

It is also demonstrated that EOS was related with AID. And we observed that except for the reduction of granulocytes and decrease of POX activity, EOS increased abnormally in marrow of RIRH patients. Those EOS were observed to adhere, capture or swallow haemocytes, which was related to serum IL-5 level, which can stimulate the production of EOS. The expression of MR, secretions of IL-12 and IL-17A mediated by EOS, and the relationship between EOS and CsA therapy may suggest that cellular immunologic response induced by EOS plays an important role in the chronic inflammatory reaction in marrow of RIRH patients. The expression of Fc γ II R and secretion of anti-human IgG induced by specific granules of EOS also indicated that EOS may participate in the process of humoral immunologic response induced by autoantibody and the pathodamage of hematopoietic cell.

We also found that HLA-B27 level and MHC-I expression increased abnormally in some RIRH patients, and lymphocyte activation and attack mediated by lymphocyte to destroy haemocyte could be found in marrow of these patients. Also HLA-B27

gene expression is related to disease progressing. Further studies are still required to verify whether virus infection could activate HLA-B27 gene structure modification and T lymphocyte receptor combination to induce CTL activation.⁵⁻⁸

Glucocorticoids can suppress the biological functions of different immunocytes. In present study we found M ϕ was distorted and miopragia, and the secretion of plasma cells was also suppressed, but with slight suppression of CTL activity. So we consider it was meaningful and important to control infection while glucocorticoids treatment associated with CsA therapy are administered in time, with the purpose to suppress Th cells activity and relieve cellular immunological damage. In addition, like other rheumatism, RIRH patients always suffer from vasculitis and other inflammations.⁹⁻¹⁰ For patients with PLT > 60 $\times$ 10⁹/L and without hemorrhagic tendency, salvia miltiorrhiza associated with safflower therapy could improve hemopoietic microenvironment of marrow and promote the metabolism of inflammatory factors, which can promote the recovery of patients.

In conclusion, RIRH was related to autoimmune disorder. Autoantibody and specific autoreactive lymphocytes were produced in marrow, leading to the hypersensitivity and pathologic damage. Those hematocytes with mutated membrane antigen or even normal hematocytes could serve as target cells in the process of autoimmunologic damage. Tissue basophils, EOS, DC, M ϕ and CTL as well as other immunocytes may participate in the pathologic process, and play a role in the resistance to myeloid element. These changes are consisted in up-regulation of cytokines and molecules, the metergasis of immunocyte, and the abnormality of HLA-B27 gene expression.

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