Evaluation of immunological criteria for rheumatoid arthritis

A. HAVRYLUK[1], R. BILYY[2], J. TOLSTIAK[1], I. KRIL[1], M. SYNENKA[1], J. ZĄBEK[3], A. PALACZ[3], J. BOGACZEWICZ[4], V. CHOPYAK[1], R. STOIKA[2]

[1]Department of Clinical Immunology and Allergology, Lviv National Medical University, Lviv, Ukraine; [2]Department of Regulation of Cell Proliferation and Apoptosis, Institute of Cell Biology, National Academy of Sciences of Ukraine, Lviv, Ukraine; [3]Department of Microbiology and Serology, Institute of Rheumatology, Warsaw, Poland; [4]Department of Dermatology and Wenerology, Medical University, Lodz, Poland

Abstract

Measurement of specific autoantibodies in RA patients cannot be considered as a universal marker of this immunopathological process. Generally accepted laboratory immunological testing algorithm is not sufficient for RA patients. There is no correlation between the expression of early and late markers of lymphocyte apoptosis.

In RA patients there is a positive correlation between the titer of lectin-induced agglutination and expression of lymphocyte activation markers. Evaluation of lectin-induced apoptosis of lymphocytes allows early detection of RA and improvement of chemotherapy scheme.

Key words: *rheumatoid arthritis, autoantibodies, markers of lymphocyte apoptosis and activation, lectin-induced agglutination.*

(Centr Eur J Immunol 2009; 34 (3): 176-181)

Introduction

Rheumatoid arthritis (RA) is a chronic systemic disease primarily of the joints, usually polyarticular. It is marked by inflammatory changes in synovial membranes and articular structures, and by muscle atrophy and bone rarefaction. In late stages of pathological process, deformity and ankylosis develop. The main cause of RA development stays unknown, however autoimmune mechanisms have been postulated. The term "autoimmune disease" is used for the conditions in which the autoimmune process contributes significantly to the pathogenesis of the disease. Criteria for the designation of autoimmune disease in human include the relevant antibodies or cell-mediated immunity operating under physiological conditions. Human diseases associated with autoantibody production include both organ-specific conditions and disorders of more generalized character. The overlaps are generally within the same category of disease, as seen in patients with organ-specific conditions or multiple "connective tissue" diseases (ex. patients with RA and systemic lupus erythematosus) [1-3].

RA is an autoimmune disease that involves in pathogenesis T-lymphocytes, B-lymphocytes, monocytes/macrophages, dendritic cells, synoviocytes, and endothelial cells [4, 5]. Performed investigations demonstrated multifuctional role of T-cells in RA. In addition to autoreactive T-cells, other regulatory T-cells may be involved. However, the mechanism of accumulation and expansion of autoreactive T-lymphocytes stay poorly studied. Limited data are available regarding functional relationships between different T-lymphocytes in RA. Activation-induced cell death (AICD) is an important mechanism that can be used by the immune system to eliminate peripherally activated lymphocytes, maintain immune cell homeostasis and peripheral tolerance in the immune system. Previous data suggest that a failure of T-lymphocytes apoptosis is involved in RA pathogenesis [5-9].

The results of our previous studies [10-13] have demonstrated that the apoptotic cells possess an increased level of α-D-mannose and β-D-galactose-rich glycoproteins in their plasma membrane, and appropriate carbohydrate-binding proteins lectins can be used for identification of apoptotic cells in culture. Changes in glycoproteins' expression were detected as early as in 6-12 hours after apoptosis induction. This article summarizes the results of utilization of available laboratory immunological methods of inflammatory

Correspondence: Anna Havryluk, Danylo Halytskyi Lviv National Medical University, Department of Clinical Immunology and Allergology, 79010 Pekarska st. 69b, Lviv, Ukraine. Phone/fax number: +38 03 22 75 61 42. Email: neyriane@ukr.net

changes in patients with RA and new such methods for studying of RA development in patients observed at the Department of Clinical Immunology and Allergology, Lviv National Medical University (Lviv, Ukraine) and at the Department of Serology and Microbiology, Institute of Rheumatology (Warszawa, Poland). Biological and medical interpretation of those data is presented.

Material and Methods

Patients

19 clinically normal donors (8 men and 11 women) and 37 RA patients (17 men and 20 women) were under investigation. Their age ranged from 18 to 43. Normal controls had no chronic or acute diseases in the past 6 months. Active RA patients who had more than six swollen and nine tender joints, as well as elevated C-reactive protein or erythrocyte sedimentation rate were selected. Peripheral blood was withdrawn in heparinised tubes and blood serum was prepared.

Detection of antibodies

Antibodies to nuclear antigens (ANA) were detected using indirect immunofluorescence test (Hep-2 cell culture, Immuno Concepts USA), diagnostic range 1 : 80. Rheumatoid factor (RF) was detected using ELISA-test (Human, Germany). Antibodies to phospholipids (antibodies to cardiolipins [aCL], antibodies to Annexin V and antibodies to β2-glycoprotein-1 [β2-GP-1] were measured by solid-phase immunoassays (ELISA) and antibodies to cyclic citrulline peptide (CCP) were measured by Euroimmun Kit (Germany) solid-phase immunoassay (ELISA).

Detection of cell surface markers

Differentiated phenotype CD 3, CD 4, CD8, CD 16, CD 22 markers, their activating markers (CD 71, CD 25, CD HLA-DR), and CD 95 marker of apoptosis were detected in isolated peripheral blood lymphocytes (standart Kits used monoclonal antibodies were purchased from principle of method – the human lymphocytes joints the particles cover with monoclonal antibodies to CD3, 4, 8, 16, 22, 25, HLA-DR, 71, 95. "Stable diagnostic kit based an monoclonal antibodies to detection subpopulation T- and B-lymphocytes" produce of State Medical Institute (Department of Allergology and Immunology) Vitebsk, Byelorussia HB YY YHH: 300002704.

Lectin-induced agglutination analysis (Agglutest)

Lymphocytes from healthy donors and RA patients were isolated from peripheral blood by centrifugation in Ficoll-Verografin density gradient. Cell viability was controlled by trypan-blue (0.1% w/v solution) exclusion test, and cell were counted in hemocytometric chamber under light microscope. The necrotic cells were detected by fluorescent microscopy using propidium iodide and ethydium bromide staining, while the apoptotic cells were detected by evaluating cell nuclei condensation and fragmentation using DAPI staining. Previously developed Agglutest [14] was used for semi-quantitive apoptosis measurement. Briefly, 20 µl of cell suspension that contained 2×10^7 cell/ml were added to 20 µl of specific lectin solutions (dilutions ranged from 1,000 µg/ml to 3.5 µg/ml) in 96-well U-shaped immunological plastic plate, and were kept for 60 min at room temperature. Cell agglutinates were scored by Epson transmissive scanner using 3,200 dpi resolution.

Statistical analysis

All experiments were carried out in triplicate and repeated at least 3 times. Significance in a typical experiment was assessed by Student's t-test. The level of significance was set at 0.05 [15].

Statistical analysis concerning autoantibodies was performed using Statistica software version 6.0 (Statsoft, Poland). As laboratory data did not fit a Gaussian distribution according to Shapiro-Wilk test, all results were expressed as mean, median (50^{th}) and interquartile range (25^{th}-75^{th}), standard deviation of the mean, and non-parametric tests were used to test statistical significance. To avoid errors inherent in repeated application of Mann-Whitney *U* tests, the Kruskal-Wallis test was performed to make simultaneous comparison of the assay data from each group, and, to determine whether there was a significant variation in the medians of the groups analysed.

Results and Discussion

We have applied two basic methodological approaches for studying autoantibodies at inflammation process: 1) internationally accepted approach used for clinical testing of rheumatological diseases; 2) general immunological testing of inflammation process. Besides, we used a novel approach that was recently developed by our team [10], and is based on measurement of early lectin-induced apoptosis in peripheric blood lymphocytes of RA patients.

The results of measurement of marker autoantibodies in the sera of RA patients are presented in Table 1 and significance of interrelation between of RA patients vs. blood donor group is presented in Table 2.

Thus, the part of patients with autoantibodies against nuclear structures and phospholipid fragments among RA patients under study is very little (Table 1). There is no need in statistical analysis in order to demonstrate that those laboratory tests are useless for confirmation of diagnosis in clinics, as well as for estimation of the activity of pathological process. Although one can find in literature detection of that group of autoantibodies in patients suffering of systemic diseases of the connective tissue, including RA patients [16-20], our data indicate that such measurement alone is not sufficient for distinct diagnosis. Other investigators also stated that some of the detected autoantibodies (including

Table 1. Marker autoantibodies in the sera of 37 RA patients

	Antibodies	Cut off values	Number of positivity
Non-specific for RA	antibodies to nuclear antigens (ANA) – IIF – Hep-2	positive at titer 1/80	12 positive (32.4% /+/) 25 negative
	antibodies to cardiolipin (IgG)	10 U/ml*	8 positive (**21.6%** /+/) 29 negative
	antibodies to Annexin V (IgG)	15 U/ml**	4 positive (10.8% /+/) 33 negative
	antibodies to β2-GP-I (IgG)	10 U/ml**	5 positive (13.5% /+/) 32 negative
Specific for RA	rheumatoid factor (RF-IgM)	34 IU/ml	21 positive (56.7% /+/) 16 negative
	antibodies to CCP (IgG)	10 RU**	14 positive (37.8% /+/) 23 negative

Cut off values was expressed in: * U/ml GPL; ** relative units (RU).

Table 2. Expression of selected serum autoantibodies in population of the RA patients and clinically healthy blood donors (control)

Autoantibody	RA patients (n = 37) $\bar{x}$ ± 1SD	Control donors (n = 19) $\bar{x}$ ± 1SD	Statistical significance (p)
Anti-nuclear (ANA)	192.16 ± 300.58	40.52 ± 37.04	**0.00007***
Anti-cardiolipin	19.54 ± 18.93	12.45 ± 1.63	0.81
Anti-β2 GP-I	0.0873 ± 0.129	0.068 ± 0.01	0.16
Anti-annexin	2.860 ± 15.46	0.004 ± 0.003	0.0000*
RF-IgM	0.370 ± 0.389	0.038 ± 0.023	0.0001*
Anti-CCP	13.458 ± 35.70	0.025 ± 0.017	0.00001*

* difference statistically significant.

rheumatoid factor and antinuclear antibodies) possess poor diagnostic significance [21-22]. They suggested that the measurement of rheumatoid factor could serve only for confirmation of diagnosis of RA joint inflammation. However, in general population the sensitivity of latex test for that disease is 80% and its specificity is about 40% [21, 27], whereas sensitivity of the ELISA-CCP test reaches also up to 80% but specificity is even higher (> 95%) then for RF.

The level of antinuclear antibodies measured by their IIF study does not correlated with changes in pathological process and with enhancement of clinical manifestation [17-19]. It is probably that blood serum of RA patients contain besides the autoantibodies with high specificity and avidity that can be detected by ANA-Hep-2 method, also a pool of autoantibodies with low avidity that can mask their detection. Changes in experimental conditions, especially of ionic strength, can cause an additional elimination of those masking autoantibodies that affects differentiated diagnostics of specific autoantibodies. It should be stressed that the indications for the diagnostic tests contain a note that the tests should be used mostly for measurement of autoantibodies with high specificity and avidity that are characteristic only for the stage of the disease manifestation [22, 24].

The appearance of antiphospholipid antibodies, including anticardiolipin (aCL) and anti-β2-glycoprotein-1 (β2-GP-I) antibodies is related to a specific set of clinical signs (artherial and venose thromboses, thrombocytopenia, livedo reticularis) celled anti-phospholipid syndrome (APS). Recently, they also attached non-specific clinical signs, such as anemia, neurological disorders, and nephropaties. Their expression in high titers is associated mostly with anti-phospholipid syndrome [25, 26]. The results o our study demonstrate moderate (108 – 21.6%) appearance of those APS indicators in RA patients.

The immunological laboratory diagnostics at RA is a multilevel one. Besides detecting autoantibodies, it is also important to measure level of non-specific immune factors that might characterize in more detail a degree of activity of the pathological process [5, 9]. We defined percentage interrelations between the main lymphocyte populations

Table 3. Percentage interrelations between the main lymphocyte populations and subpopulations in RA patients

Lymphocyte phenotype	RA patients (n = 37)	Control patients (n = 19)	T	p
CD3+	52.027 ± 1.005	48.000 ± 1.060	2.522	< 0.05
CD4+	27.730 ± 0.836	24.737 ± 0.764	2.317	< 0.05
CD8+	20.649 ± 0.937	14.474 ± 0.821	4.317	< 0.001
CD16+	20.919 ± 1.173	13.632 ± 0.650	4.267	< 0.001
CD22+	23.054 ± 0.852	20.842 ± 0.758	1.689	< 0.097

Table 4. Percentage indicators of activation markers in lymphocytes populations of RA patients

Lymphocyte phenotype	RA patients (n = 37)	Control patients (n = 19)	T	p
CD25+	24.784 ± 1.280	14.316 ± 0.970	5.446	< 0.001
CD71+	21.459 ± 1.362	13.158 ± 0.706	4.207	< 0.001
CD95+	19.946 ± 0.964	12.421 ± 0.842	5.319	< 0.001
CD HLA-DR+	22.000 ± 0.964	13.105 ± 0.582	6.294	< 0.001

Table 5. Apoptotic markers in lymphocytes of RA patients

Titer of lectin-induced agglutination in RA patients	RA patients (CD 95+) (n = 37)	Control (CD 95+) (n = 19)	T	p
8, 7, 5 (n = 17) high	16.300 ± 0.128	12.420 ± 0.199	3.675	< 0.001
2, 1, 0 (n = 20) low	20.180 ± 0,204	12.420 ± 0.199	10.218	< 0.001

and cell markers reflecting activation of immunocompetent cells (Tables 3, 4).

It was found that during RA start the main role in the impairment of joint tissues is played by activated T-lymphocytes and macrophages which synthesize proinflammatory cytokines – TNF-α and IL-1,6,8,12 [3, 9, 16, 27]. As a result of those processes an inflammatory reaction is developed. At later stages of the disease development, cells are stimulated that participate in destruction of joint cartilage and bone, as well as an enhancement of development of new vessels, expression of adhesion molecules on the endothelial cells, elevated activity of cyclo-oxygenase 2 and production of prostaglandins, metaloproteinases, complement components, lysosomal enzymes and reactive oxygen species takes place [23].

Table 3 demonstrates statistically significant elevation of content of all populations and subpopulations of T-lymphocytes in RA patients that indicates development of inflammatory reaction.

Table 4 demonstrates statistically significant increase in the content of activation lymphocyte markers in RA patients. It can be seen best of all when early activation marker CD71+ is determined, since that marker appearance is a reflection of the inflammatory process in RA patients [28]. A significant increase in the content of late activation markers such as CD25+ and CD HLA-DR+ in RA patients indicates an enhancement of production of pro-inflammatory cytokines, as well as activation of B-lymphocytes with following synthesis of antibodies. It should be stressed that the patients were observed at peak of appearance of clinical signs before the start of basic therapy application. Thus, although determination of lymphocyte populations and subpopulations is considered to be a generally accepted immunological testing, its role in defining a leading effector mechanism at RA development is not sufficient.

For detection of cytotoxic impairment of lymphocytes in RA patients, we have carried out measurement of apoptosis using lectin-agglutination test [29, 30]. Such study allows to characterize relatively early stages of apoptosis, since it can demonstrate changes in glycoproteins of lymphocyte plasma membrane. An appearance of late apoptosis was measured on the basis of expression of CD95+ (Fas-receptor). The results obtained are presented in Table 5.

These results (Table 5) indicate reverse proportional dependence between the indicators of early and late apoptosis in RA patients, namely it was found that patients with high

Table 6. Comparison of immunoregulatory index and agglutination indicator in lymphocytes of RA patients

Lymphocyte phenotype	RA patients (n = 37)	Control patients (n = 19)	T	p
Agglutination indicator	4.297 ± 0.537	0.474 ± 0.118	5.051	< 0.001
CD 4/CD 8	1.450 ± 0.088	1.805 ± 0.112	–2.425	< 0.05

titer of lectin-induced agglutination of lymphocytes is characterized by relatively low amount of lymphocytes that expressed Fas-receptors.

The test of lectin-induced agglutination of lymphocytes allows defining relative number of cells which enter early apoptosis in RA patients. It was found that the titers of agglutination correspond both elevated indicators of populations and subpopulations of lymphocytes and the level of expression of the main activation markers (Tables 3, 4, 6). It is known that apoptosis at autoimmune diseases is realized via receptor-mediated pathway using Fas-Apo system, phosphatydylserine externalization and changes in plasma membrane glycoproteins [7]. Apoptosis role at autoimmune diseases stays poorly studied, since those diseases usually have unclear etiology and numerous pathogenetic mechanisms [29, 31]. For example, at systemic lupus erythematosus (SLE) the main antigenic material is nucleosomes, that appear as apoptotic material in circulation and induce immune response [13]. The role of lymphocyte apoptosis playing principal role in pathogenesis of autoimmune diseases is also poorly studied [13, 16, 29]. Tang X. et al. [8] suggested that T-cell apoptosis in RA patients is suppressed. However, it was shown that the mechanisms for programmed cell death appear to be intact in synovial infiltrating cells. The apoptosis of peripheral lymphocytes also appeared to be normal in RA patients. T-lymphocytes in RA patients were found to be apoptosis-sensitive [8, 31]. We consider that switching on lymphocyte apoptosis indicates the start of pathological process that is important for disease prognosis, especially taking into account that autoimmune disease starts with disturbance of cellular (cytotoxic) and not humoral immune effector mechanism [1].

Thus, lectin-induced agglutination analysis was proved to be effective for measuring apoptosis in isolated lymphocyte suspension. It should be also noted that such approach for apoptosis study is rather convenient, since it is characterized by low cost of analysis and relatively high speed (approximately 1 hour).

References

1. Gołąb J, Jakóbisiak M, Lasek W. Immunologia. Wydawnictwo Naukowe PWN, Warszawa 2002; p. 567.
2. Smerling RH, Delbanco TL (1991): The rheumatoid factor: an analysis of clinical utility. Am J Med 91: 528-534.
3. Ziółkowska M, Maśliński W (2003): Laboratory changes on anti-tumor necrosis factor treatment in rheumatoid arthritis. Curr Opin Rheumatol 15: 267-273.
4. Bany U, Gajewski M, Księżopolska-Pietrzak K et al. (1999): Expression of mRNA encoding muscarinic receptor subtypes in neutrophils of patients with rheumatoid arthritis. Ann N J Acad Sci 876: 301-304.
5. Majanskyj NA (2004): Neutrophils apoptosis internal pathway and apoptotic effect mechanisms of granulocyte colony-stimulating factor. Immunologija 6: 329-335.
6. Dugawson CE, Nelson JL, Koepsell TD (1990): Evaluation of the 1987 revised criteria for rheumatoid arthritis in a cohort a newly diagnostic female patients. Arthr Rheum 33: 1042-1046.
7. Lawen A (2003): Apoptosis – an introduction. BoiEssays 25: 888-896.
8. Tang X, Yocum DE, Dejonghe D et al. (2004): Increased activation-induced cell death in peripheral lymphocytes of rheumatoid arthritis patients: the mechanism of action. Immunology 112: 496-505.
9. Jaremko OB (2004): Early rheumatoid arthritis (diagnostics and treatment). Mystetstvo likuvannia 3: 38-51.
10. Bilyy RO, Antonyuk VO, Stoika RS (2004): Cytochemical study of role of alpha-d-mannose- and beta-d-galactose-containang glycoproteins in apoptosis. J Mol Histol 35: 829-838.
11. Bilyy R, Kit Y, Hellman U (2005): In vivo expression and characteristics of novel alpha-d-mannose-rich glycoprotein markers of apoptotic cells. Cell Biol Int 29: 920-928.
12. Bilyy RO, Stoika R (2007): Search for novel cell surface markers of apoptotic cells. Autoimmunity 40: 249-253.
13. Stoika RS, Bilyj RO. Novel glycoprotein markers on surface of apoptotic cells: potential diagnostic role. 2005.
14. Stoika RS, Bilyj RO, Antoniuk VO (2006): Basic agglutination method for rapid detection, isolation and characteristics of apoptotic cells patent RST No 60 731, 768 in the USA Patent Office.
15. Derkach MP, Gumetskyj RJ, Chaban MJ (1977): Variation statistics course. K Vyshcha Shkola 207.
16. Carp HJ. Autoantibodies and pregnancy loss. In: Shoenfeld Y, Gershwin E, Meroni PL (eds.). Autoantibodies. Elsevier 2007; pp. 809-814.
17. Casiano C, Tan E (1996): Antinuclear autoantibodies: probes for defining proteolytic events associated with apoptosis. Mol Biol 23: 211-216.
18. Matsuura E, Dier KJ, Lopez LR. beta2-glicoprotein I autoantibodies. In: Shoenfeld Y, Gershwin E, Meroni PL (eds.). Autoantibodies. Elsevier 2007; pp. 687-693.
19. Emlen W, Onell L (1997): Clinical significance of antinuclear antibodies; comparison of detection with immunofluorescence and enzymelinked immunosorbent assays. Arthritis Rheum 40: 1612-1618.
20. Invernizzi P. Antinuclear antibodies: general introduction. In: Shoenfeld Y, Gershwin E, Meroni PL (eds.). Autoantibodies. Elsevier 2007; pp. 313-322.
21. Ward MM (1999): Laboratory tests in connective tissue diseases. Medycyna po Dyplomie 8: 45-52.
22. Ząbek J (2005): Standardization of the methods of "markers" autoantibody assessment in the ECFS projects – aims and real achievements. Reumatol 43: 179-182.
23. Angelis V, Meroni PL. Rheumatoid factors. In: Shoenfeld Y, Gershwin E, Meroni PL (eds.). Autoantibodies. Elsevier 2007; pp. 755-762.

24. Ząbek J, Palacz A, Krzewska I (2006): Preliminary data concerning methodology of assessment of serum autoantibodies not included into CTD-s diagnostic criteria. Reumatol 44: 293-297.
25. Zimmermann-Górska I (2006): Classification criteria for APS syndrome – next modification. Pol Arch Med Wewn 5: 396-400.
26. Zimmermann-Górska I (2005): Twój Magazyn Medyczny. Reumatologia. Wydanie specjalne. I Ogólnopolska Konferencja: "Zespół antyfosfolipidowy". X 3/152.
27. Kontny E, Ziółkowska M, Ryżewska A (1999): Protein kinase pathway is critical for production of pro-inflammatory cytokines (TNF-alpha, IL-1beta, IL-6). Cytokine 11: 839-848.
28. Asherson RA, Cervera R (2003): Antiphospholipid antibodies and infections. Ann Rheum Dis 62: 388-393.
29. Kobayashi K, Kishi M, Atsumi T et al. (2003): Circulating oxidized LDL forms complexes with beta2-GP-I: implication as an atherogenic autoantigen. J Lipid Res 44: 716-726.
30. Matsuura E, Dier KJ, Lopez LR (2007): beta2-glicoprotein I autoantibodies. In: Shoenfeld Y, Gershwin E, Meroni PL (eds.). Autoantibodies. Elsevier 2007; pp. 687-693.
31. Dejaco Ch, Duftner C, Grubeck-Loebenstein B (2005): Imbalance of regulatory T-cells in human autoimmune diseases. Immunol 117: 289-300.