Evaluation of serum natural autoantibodies reaction in different hematological disorders with prospective view to their probable utilization in predictive medicine

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Abstract:
BACKGROUND: There are some antibodies which are present in healthy individuals without any former exposure to foreign antigens; they are known as natural autoantibodies (NAAbs). In recent years, it was shown that they probably contribute to the homeostasis of the whole body and might be present before beginning of some diseases. Thus, as new biomarkers, they are promising factors to diagnose diseases.

MATERIALS AND METHODS: In this study, we drew upon samples of 924 individuals (600 controls and 324 cases) with underlying diseases of anemia, polycythemia, leukocytosis, thrombocytopenia, thrombocytosis, and pancytopenia. For detection of NAAbs against red blood cell, plasma samples were incubated with their own red cell suspension in 4°C for 18 h. Then, positive samples were evaluated for antibody screening and titration.

RESULTS: Fifty-two (8.6%) controls and 58 (17.9%) cases showed positive reaction (Pv < 0.001). The prevalence of positive antibody screens among auto-positive controls was 53% and 100% among cases; moreover, strength of antibody screen reaction had a mean rank of 22.5 in controls and a mean rank of 38.5 in cases (Pv < 0.001). A significant relation was also observed between ABO blood group and prevalence of NAAbs in controls but not in cases (Pv < 0.05).

CONCLUSION: The prevalence and potency of NAAbs increased along with hematological changes; moreover, the antibody reactions’ pattern and titration showed significant differences between the two groups and these may be useful as biomarker for monitoring and prediction of some hematological diseases.

Keywords:
Hematological disorders, natural autoantibodies, predictive medicine

Introduction

It is well established that natural antibodies (NABs) are present in healthy individuals without any former deliberate immunization, but generally, are able to react with a widespread range of antigens from self-molecules (e.g., phospholipids, serum proteins, cellular components, insulin, and thyroglobulin) to exogenous antigens.¹,² For a long time, NABs had been considered as an unimportant part of immunity, but recently, more research on NABs has clarified surprising and even controversial physiologic and pathological aspects. When for the first time, Grabar, in 1975, suggested a physiologic function for NABs, clearance of cell debris, has...
substantially put attention to these multifunctional Abs, and until now broad studies have indicated significant roles in not only protection against exogenous antigens and facilitating apoptosis but also in atherosclerosis, neurodegenerative disorder, and malignancy, that gave rise to present knowledge in their necessary role in maintenance of immune homeostasis.\textsuperscript{[3,4]} Nowadays, their roles have been clarified including cancer-specific apoptosis, NAbs against phosphorylcholine as a strong protection marker in atherosclerosis, DC differentiation, and inflammation control mediated by antileukocyte NAbs.\textsuperscript{[5-9]} It has been also suggested that polyreactivity of NAbs probably gives them a beneficial property that leads to formation of a massive, dynamic network controlling whole body homeostasis which in turn actively causes physiologic autoimmunity.\textsuperscript{[10]} This intellectual system that is permanently affected by internal and external stimulus, purposefully prevents stimulation of pathologic autoimmunity.\textsuperscript{[11-14]} Accordingly, a hypothesis, termed “Immunological Homunculus,” considered natural auto antibodies as the mirror of organism.\textsuperscript{[12]} This point of view proposed that any excessive changes in physiologic condition of body in terms of quantity and intensity will affect the NAbs content. Then more accomplished results based on more observation like microarray test (antigen-chip) have caused some of these NABS to be considered as “biomarkers”. So they can prepare an early diagnosis possibility of “predictive medicine” at the early stage of disease, making an opportunity for identification of pathologic changes in the body.\textsuperscript{[15-16]} My colleagues have previously shown that the mean immune reactivity of serum antibodies with 24 human autoantigens increased with advancing age, which could be because of the increase in the level of pathological antibodies.\textsuperscript{[3]} Here, we utilize immune hematology methods for NABS screening against red blood cells (RBCs) surface antigens under physiologic changes of blood in two populations, healthy blood donors (controls) and hematological patients’ group (cases).

**Materials and Methods**

**Study design and sample preparation**

The study population included the total number of 924 individuals (600 controls and 324 cases) including relative polycythemia, Hb: >18 g/dl, n = 131; anemia, Hb: <10 g/dl, n = 101; leukocytosis, white blood cell >20000 mcl, n = 47; thrombocytopenia, plt: <100,000 mcl, n = 21; thrombocytosis, plt: >450,000, n = 14; and pancytopenia, n = 12. Criteria for selecting this number of samples were based on achievement to an equal number of auto-positive samples in two groups. Blood specimens were obtained from 600 normal individuals and 324 cases into two groups (total 924). Blood samples were collected under the supervision of a physician. Ethylenediaminetetraacetic acid blood samples of controls were collected from March 2015 to November 2015 in Tehran and Karaj Blood Donor Centers. The cases’ samples were collected in Iran Blood Transfusion Organization (IBTO) Hematology Reference Laboratory. The blood donors were informed about the study aim and procedure, and written consent was obtained by the medical interviewer. All the protocols were confirmed by the Ethical Committee of Medical Faculty of Tarbiat Modares University.

Since we were looking for cold reactive natural IgM antibodies with the inability of complement activation, instead of DAT test, all samples with overnight incubation at 4°C underwent a test for natural autoantibodies; then for samples which had shown positive reaction at first stage supplementary tests including antibody screening and specificity test were performed. Standard serologic procedures and manufacturer’s directions were followed as described in the AABB Technical Manual.\textsuperscript{[17]} ABO and Rh(D) blood typing was performed with reagents purchased from Diagast (251, Av. E. Avinee-BP, 9 59374 Loos France). The antibody screening test was performed with three cells’ reagent by tube testing at 4°C in saline media. The tests of antibody specificity were performed using Oladult, Oclord, autologous, and ABO compatible cells.\textsuperscript{[17,18]} All different cells including O cells, panel cells, cord blood Oi cells, and A1 and B cells were obtained from IBTO Immunohematology Reference Laboratory. To ensure, in the case group, the prewarm indirect antiglobulin test (IAT) was performed with polyspecific anti-immunoglobulin G + Anti-C3d and enhancing agent (LISS), any positive reaction at 37°C led to the removal of the sample. Anti-C3d + Anti IgG (polyspecific) antiglobulin reagents were also obtained from Diagast. Antibody titrations were performed with serial dilutions of the serum in physiologic saline at the same temperature as the antibody screening tests.\textsuperscript{[19]}

**Statistics**

Computer software SPSS 16 (IBM, SPSS Inc., Version 16.0, Chicago) and also GraphPad Prism (version: 5.01, GraphPad Software, Inc., USA) were used for data analysis. Analysis of blood groups’ prevalence in positive samples was carried out using Chi-Square; moreover, Mann–Whitney and binomial tests respectively were carried out to measure mean rank of antibody screening results and natural autoantibodies’ (NAABS) prevalence rate in different groups. \( P < 0.05 \) was interpreted as statistically significant.

**Result**

**Frequency and titration of natural autoantibodies**

Both prevalence and titration of NAABS increased among case group; moreover, binomial analysis showed that intended ratio of NAABS in control group significantly differed from case group (\( P = 0.001 \)).
Potency of natural autoantibodies reaction

According to antibody screening results, the strength of NAAbs reaction in case group was significantly higher than normal population \((P = 0.001)\) [Figure 1]. Moreover, the number of samples with NAAbs in case group was higher than in the controls [Table 1]. In antibody screening for case and control groups of this study, the potency of antibody in control group was very low and not enough for titration, but in case group, the potency of antibody was very higher even up to 1024-fold titration and also as has been showed in Figure 1. Pan reactivity of the patient samples was significantly increased compare to control group.

Incidence of natural autoantibodies reaction in different hematological disorders

A significant relationship was observed between the incidence of reaction and type of disease in most of the abnormal cases as it has been shown in Table 2; also the highest percent of reactions was observed in pancytopenia both in terms of strength and incidence rate.

Relation between natural autoantibodies and ABO blood group

Among whole positive samples in control group, blood groups were as follows: A = 46.1\% (24 cases), O = 23\% (12 cases), B = 21\% (11 cases), and AB = 9.9\% (5 cases). Distribution of ABO blood group in case group compare to control was not significant;[20] but in control group, the A blood group showed a significant increase in compare the normal population \((P = 0.01)\).

Discussion

In this study, we investigated sera for NAAbs reaction in 924 individuals in two populations of healthy blood donors as controls and hematologic patients as cases. NAAbs are the capacity of immune system to respond to foreign antigens. It has been shown that the amount of these antibodies increases with age and responsible for the increased susceptibility of the elderly to infection and cancer.[21] The CD5+ B-1 cells are responsible for the secretion of natural autoantibodies with no stimulation from the early stages of embryogenesis.[22]

In our previous study, we demonstrated that there is association between human B-1 cell frequency and aging.[23] As well as reactivity of natural autoantibody with auto-antigens changed in different age intervals among healthy individuals and increased in the elderly people. Regarding the crucial roles of natural autoantibodies in maintaining the body homeostasis, these variations might represent the immunological and pathological condition in the elderly.[2] Concentration of total IgG and IgM was determined in different age intervals. Concentration of IgG has been shown increasing trend along with age. On the contrary, the mean value of total IgM showed an increasing pattern from cord blood to the middle age (40 years) after which it dropped, while, as noted above, IgG concentration followed an increasing trend all the way from infants to the elderly. The decrease in IgM level showed positive

![Intensity of reaction](image)

**Figure 1:** Strength of antibody screen reaction had a mean rank of = 22.5 in controls and a mean rank of = 38.5 in cases \((Pv = 0.001)\)

| Table 1: The cold reaction of red blood cells with self-serum significantly increased in case group compare to control and subsequently in alloreactivity and titration |
|---|---|---|
| Auto check | Control, \(n(\%)\) | Case, \(n(\%)\) |
| Total | 600 | 324 |
| Auto-positive | 52 (8.6) | 58 (17.9) |
| Auto-negative | 548 (91.4) | 266 (82.0) |
| Ab screening and titration \((n=30)\)* | | |
| Screen positive | 16 (53) | 30 (100) |
| Screen negative | 14 (47) | 0 |
| Titration | All=2 | 2–1024 |

*Only those samples were selected for Ab screening which had at least a minimum + 1 reaction

| Table 2: Distribution of positive reaction in different hematopathological conditions, as shown in table, the most association was found with pancytopenia and the least with thrombocytopenia |
|---|---|---|---|---|---|---|
| | Pancytopenia, \(n(\%)\) | Trombocytopenia, \(n(\%)\) | Trombocytopenia, \(n(\%)\) | Leucocytosis, \(n(\%)\) | Anemia, \(n(\%)\) | Polycytemia, \(n(\%)\) |
| Total | 12 (100) | 21 (100) | 14 (100) | 47 (100) | 101 (100) | 131 (100) |
| Positive reaction | 6 (50) | 8 (38) | 1 (7) | 12 (25.5) | 11 (10.8) | 20 (15.2) |
| Negative reaction | 6 (50) | 13 (62) | 13 (93) | 35 (74.5) | 90 (89.2) | 111 (84.8) |
| \(P\) | <0.001 | <0.001 | >0.05 | <0.001 | >0.05 | <0.001 |
association with the frequency susceptibility of the elderly to autoimmune disease and cancer. [24] Thus, as expected, we apparently detected the increased levels of NAAbs at least in a part of healthy population that had exceeded micromolar level. Therefore, on the one hand, it can be said that one of the reasons for success of immune system largely depends on proper repertoires of NAAbs against carbohydrates that stand for their various and essential function in all tissues, [25] but on the other hand, affecting internal and external stimuli and regulation mechanisms should be considered although they are little known. [11] Based on prior research, a blood group shows more heterogeneity, subgroups even show more genes than B blood groups. Therefore, it likely represents which more anti-A reflects variety of heterogeneous antigens. [25, 26] It should be noted that the term “polyreactivity” of NAAbs does not mean lack of specificity rather a tendency to a set of particular epitopes that give them so many essentially biological roles. [27] According to observation following immunoculus theory, NAAbs are reflectors of body inner picture in a molecular scale, and if any alteration for any reasons occurs in body condition, it will be reflected in the NAAbs content. [12-16] RBCs transfusion in patients can lead to the remodeling of NAAbs autoreactivity. [28] NAAbs are able to identify Damage-associated molecular patterns (DAMPs) and in fact as sensors play a protective role in immunity against oxidative epitopes. [29] Furthermore, it has been shown that NAAbs regulate immune system and a large number of NAbS have a key role in tissue homeostasis. Immunosurveillance mechanism has a role in cancer prevention and the presence of NAbS in normal range/activity in healthy individuals is essential for body to maintain hemostasis. [30]

In the present study, we were able to demonstrate that the reaction of sera NAAbs both in terms of incidence and intensity or potency that increases along with alteration in physiologic and pathologic changes depends on the related tissue (blood) and the most alterations were related to the group with all blood cells disorder. Exploring the fact hidden behind NAAbs as a boundary among physiology and pathology serves us to understand a part of this complex network.

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Conflicts of interest
There are no conflicts of interest.

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