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Review

Antinuclear autoantibodies in health: autoimmunity is not a synonym of autoimmune disease

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Abstract: Incidence of autoimmune diseases increases. Antinuclear antibodies (ANA) testing is a critical tool for their diagnosis. However, ANA prevalence in health increased over last decades, especially among young people. ANA in health occur in low concentrations, with prevalence up to 50% in some populations, which demands a cutoff revision. The review deals with origin and probable physiological or compensatory function of ANA in health, according to the concept of immunological clearance, theory of autoimmune regulation of cell functions and the concept of functional autoantibodies. Considering ANA titers ≤1:320 as a serological marker of autoimmune diseases seems inappropriate. The role of anti-DFS70/LEDGFp75 autoantibodies is highlighted as possible anti-risk biomarker for autoimmune rheumatic disorders. ANA prevalence in health is different in various regions due to several underlying causes discussed in the review, all influencing in additive combinations according to the concept of the mosaic of autoimmunity. Not only titer, but the HEp-2 IFA staining patterns, like AC-2, is also important. Accepting autoantibodies as a kind of bioregulators, not only upper, but also lower borders of their normal range should be determined. Not only their excess, but also lack of them or "autoimmunodeficiency" could be a reason of disorders.

Keywords: autoimmune diseases; antinuclear antibodies; antinuclear factor, functional autoantibodies; natural autoantibodies, physiological autoimmunity

1. Introduction

The incidence of autoimmune diseases (ADs) is high worldwide among both adults and children. According to various estimates, the total incidence of ADs in different countries and regions varies from 5% to almost 30%, and there is an annual increase [1-5]. This is especially typical for systemic ADs with non-organ-specific autoantibodies [1]. ADs significantly impair the quality of life of patients and often lead to severe, usually lifelong disability. They require significant costs from the health care system when diagnosed at late stage: for example, the annual costs of treating one case of systemic lupus erythematosus (SLE) with renal or neuropsychiatric complications in USA in 2013 exceeded 30 000-32 000 US $, being 6.25-6.5 times higher than the cost of treating the initial, inactive and uncomplicated phases of the disease [6]. Early diagnosis of ADs is desirable, but the process often takes a long time, due to the absence of specific symptoms in early stages of the disease in many patients [7-10]. This factor determines the relevance of the search for laboratory tests suitable for the early diagnosis and screening of ADs in order to timely prescribe appropriate treatment.

Identification of different autoantibodies is used for the diagnosis of ADs [11, 12]. Some of them are associated with specific autoimmune diseases [10, 13-15]. However, the reliability of autoantibodies as pathognomonic markers of a particular disease is far from 100%, moreover, many

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of them are observed in several different nosological entities. Their association with a certain disease sometimes requires confirmation of antigenic specificity by several different methods, therefore the general consensus is that the term “disease associations” should be replaced by “clinical relevance” of the identified autoantibodies [16].

In addition, there is a growing number of evidence that the responsibility of the immune system is not only (and even not mainly) the protection against foreign intrusions. Immune system serves as physiological sensory and analytical instrument in relation to the antigenic structure of the multicellular organism, responsible for its maintenance and even its formation. Therefore auto-recognition is regarded as a normal primary function of the immune system, which is associated with the existence of physiological autoimmunity [17, 18].

In this review we address the issue of antinuclear autoantibodies (ANA) testing, which has been detected by the reaction of indirect immunofluorescence and known since 1958 under the traditional name "antinuclear factor" (ANF) [19]. We discuss ANA presence and role in healthy individuals, the peculiarities of distinguishing between normal and pathological positive ANA-test results, the role of ontogenetic and geographical factors in ANA prevalence - in the context of the concept of physiological autoimmunity and its relationship with ADs.

2. Physiological autoimmunity and its bidirectional pathological changes

Almost all autoantibodies, including ANA, are often found not only in the sera of patients who suffer from ADs, but also in healthy persons (including those who do not develop a disease during follow-up), although in health they are usually present in low titers [19-22].

The issue of natural autoantibodies and physiological autoimmunity has acquired considerable relevance with the development of more sensitive laboratory tests, because with these methods autoantibodies to many antigens, including cell nuclei and membrane receptors, have become routinely registered in the blood and biological secrets of healthy people [23-29]. This is also true for the autoantibodies that are considered typical markers of certain ADs, for example, IgG against the basement membrane of the glomeruli, proteinase 3, myeloperoxidase, myelin basic protein etc. [30, 31].

Moreover, due to the presence of both agonistic and antagonistic effects of such autoantibodies of healthy donors on the receptors of neurotransmitters [32, 33], hormones [34, 35], autacoids [36, 37] and IgE [38], the question of their possible physiological regulatory role has long been raised. Indeed, there is growing evidence for this aspect of physiological autoimmunity [39-42].

In accordance with this assumption several fruitful scientific doctrines have been formulated in different periods, rooted in the ideas of I.I. Mechnikov who considered the immune system as a means of forming and maintaining the multicellularity of the metazoan organism [43]. These doctrines are: the doctrine of cytotoxins, the theory of autoimmune regulation of cellular functions, and the theory of immunological clearance [39, 41, 44, 45]. The term "functional autoantibodies" (which primarily relates to the autoantibodies against plasma membrane receptors) was coined in recent years and indicates the modern version of the mentioned ideas [32, 46, 47].

However, with regard to the functional properties, ANA cannot be considered an exception, since it was shown in vivo and in vitro that ANA can penetrate not only into the cytoplasm, but also into the nuclei of living cells (moreover, it occurs with the involvement of antigen binding fragments). ANA can influence gene expression, cell growth and apoptosis. Therefore, not only regulation, mediated by membrane receptors, but also repression / activation of genes through cis-regulatory elements of chromatin can be carried out by autoimmune mechanisms [44, 48-52].

It is no coincidence that there is a growing number of studies which showed that:

1. Patients with certain ADs have not an increase, but a decrease in the level of certain autoantibodies and / or the strength of antibody-mediated bioeffects, in comparison with healthy donors;
2. The level of autoantibodies decreases, rather than increases during exacerbations of some ADs;
3. Some autoantibodies are associated with a favorable outcome of the disease.

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This was demonstrated both for the autoantibodies to cell membrane receptors [42, 53-56] and for ANA [12, 57-58]. For example anti-DFS70 autoantibodies against the lens epithelium-derived growth factor are more prevalent in healthy people than in patients with ADs, and have been considered in recent years as an important marker of the lower probability of rheumatological diseases, even with ANA test being positive [59]. Moreover, since it is the nuclear autoantigen DFS70 that has been identified as a co-factor for the replication of the human immunodeficiency virus (HIV) through its interaction with the viral integrase, these autoantibodies may also have protective activity against HIV-infection [60].

The paradigm of "beneficial autoimmunity" [18, 45, 61] is also supported by the use of autoantibodies against nuclear antigens obtained from patients with ADs for the treatment of certain cancers [42, 50].

Traditionally, it is assumed in diagnostic immunology that the more autoantibodies (either damaging or functional ones) patient has, the more symptoms of the disease will be manifested [62].

But, apparently, the level of some autoantibodies in a healthy individual should be no more, but no less than the optimum [53, 63]. It has been suggested, by analogy with endocrine disorders which can equally occur both from excess and deficiency of a certain signaling molecule, that not only an increase, but also a pathological decrease in the concentration of autoantibodies may reflect and even cause pathological processes in the body [64]. Thus, autoimmunity and ADs are not synonymous terms.

Only a pathological intensity of the autoimmune reaction or its insufficient regulation causes illness. Perhaps, along with the terms that have been coined long ago to denote excessive ("allergy") and insufficient ("immunodeficiency") response of the adaptive immunity to foreign antigens, we should start using the terms "autoallergy" and "autoimmunodeficiency" to indicate diseases caused by disorders of natural self-recognition process being polarized in opposite directions [63-64].

According to the current opinion, ANA, like some other autoreactive immunoglobulins and lymphoid clones, belong to integral part of the normal functioning of the immune system [24, 28, 32, 36, 39-42, 44-47, 63-65].

Notably, that as early as 1984, N.K. Jerne [66] established the theory that the immunity is controlled and restrained by nothing more than natural autoimmunity, implying an idiotype-anti-idiotypic network of mutually recognizing antibodies and lymphoid clones. Now this theory is complemented with the data on the origin of T- regulators which derive from the differentiation of autoreactive clones with moderate affinity[67].

But the role of natural autoantibodies (including ANA) in immunity and, more broadly, in the homeostasis of the body is still not entirely clear. Since the 1980ies, some studies have appeared that demonstrated the existence of "natural" autoantibodies. They are produced by B1 cells without antigenic stimulation and are considered as the part of the innate immune system [28, 65, 68]. Their source, in particular, are the so-called CD20CD27CD43CD5 + (or CD5–) CD70– B cells, a self-sustaining population of descendants of fetal rudimentary lymphoid cells which are generated in the liver and bone marrow in early ontogenesis. They are originally predominant in serous cavities (peritoneal and pleural) and capable to settle elsewhere, including in the lamina propria of the gastrointestinal tract and inflammation foci, but only occasionally present in encapsulated secondary lymphoid organs [69].

What distinguishes natural autoantibodies is their specificity for a wide range of structurally unrelated antigens such as DNA and insulin, phospholipids and myelin basic protein, oxidized lipoproteins, etc. [70-72]. Natural autoantibodies are primarily IgM, but sometimes they belong to other isotypes [42]. The aforementioned subset of B cells, which is related to the paleo-immunity, undoubtedly play an important role, in particular in the suppression of the hypersensitivity of the mucous membranes and in the development of oral tolerance [69].

However, we consider that the entire phenomenon of physiological autoimmunity cannot be attributed only to the competence of B1 lymphocytes. After all, autoantibodies of healthy individuals often have high affinity to their targets (for example, anti-DFS70 autoantibodies and autoantibodies

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to receptors of various bioregulators). Also, natural autoantibodies can belong to immunoglobulin isotypes other than IgM [52].

Such natural autoantibodies are involved in the elimination of the body's own antigens and neoantigens which are formed during cell death or alteration of various biomolecules [22, 39, 45, 61, 63, 70]. They are credited with protective functions related to the immune clearance of certain antigens, including atherogenic lipoproteins, proteins deposited in neurodegenerative diseases, and other pathogenic autoantibodies [55, 65, 70, 75].

The assumption about the important role of natural autoantibodies and macrophages in the immune clearance was first made by the inventor of immunoelectrophoresis Pierre N. Grabar more than half a century ago [76, 77]. At that time, Mechnikov's approach to the problems of physiological autoimmunity gave rise to many ideas in the literature (mainly written in Russian and French), regarding the role of natural autoantibodies, including their possible participation in the regulation of cellular membrane permeability, intracellular content of macromolecules, in antitumor protection, and even in radioprotection [78-81].

All these approaches were associated with the indisputable conviction of immunologists prevailing at that time and documented in most authoritative handbooks, that such a large molecule as immunoglobulin can perform its functions only outside the cells, in biological fluids or on the cell surface [82].

The idea of P. N. Grabar did not require assumptions that contradicted this dogma, and immediately found supporters. It was further developed in the concept of immunochemical homeostasis by I. E. Kovalev [83]. In accordance with the main postulate of this concept, the levels of natural autoantibodies are regulated according to the principle of feedbacks by the number / availability of molecules of the corresponding autoantigens. Since the levels of expression and secretion into the extracellular space of any cytoplasmic, membrane, nuclear and other autoantigens differ little in healthy individuals, the serum levels of autoantibodies of corresponding specificity also differ just slightly. However, with the development of any disease, the picture changes, to the extent that the natural dynamics of cell populations is distorted.

From this point of view, it is important not only to measure the absolute levels of certain autoantibodies, but also to compare them with the average levels of these autoantibodies in the population of a particular region. An individual's autoreactivity (i.e. the spectra and ratios of autoantibodies of different specificity) should be also taken into account and the attention should be paid, first of all, to those autoantibodies that give positive or negative peaks against the background of the general "landscape". These principles are reflected in some approach to the immunological screening and immunodiagnostics and continues to evolve [37, 41, 50, 84].

Many targets of the naturally occurring autoantibodies, such as DNA, histones, nucleoproteins, and phospholipids, are typical components of apoptotic bodies. In this regard, there is a point of view that one of the main physiological functions of moderate autoimmunity is the elimination of apoptotic debris. Notably, that the same antigens which are abundant among the products of apoptosis (the mentioned above, as well as the products of apoptogenic proteases, agonists of cytokines receptors and chemokines receptors) are also targets of pathological autoantibodies in rheumatological ADs [63, 85, 86]. It is possible that this group of diseases will someday be designated as “autoimmune diseases with autoantibodies against the components of apoptosis” which we have already proposed [63].

Normally (possibly - just by means of physiological natural autoantibodies), the products of apoptosis are phagocyted by none antigen-presenting CD68-positive tingible body macrophages, but in the case of impaired clearance, a significant part of the debris is engulfed by antigen-presenting cells. The enhanced presentation of apoptotic autoantigens triggers an overly enhanced autoimmune response against those autoantigens (in particular, antinuclear ones), which was noted in individuals predisposed to systemic ADs in contrast to healthy ones [86].

It has been shown that immunization of mice with one of the proteins involved in the clearance of apoptotic bodies, pentraxin-3, led to the emergence of protective autoimmunity and was associated the delayed development of experimental lupus nephritis [87]. Back in the early 1980ies, it was found

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that the number of B-lymphocytes capable of recognizing double-stranded DNA, one of the main autoantigens in the ANA, is the same in the blood of SLE patients and healthy donors [88]. The process of antigen presentation and the effects of T cells on this process can determine, therefore, whether autoimmunity will be maintained within the physiological limits, or it will evolve in AD.

Thus, it is possible that natural autoantibodies may precede the appearance of pathological autoantibodies. An increased level of polyreactive B cells is found in patients with ADs [88, 89]. Mature naive B cells in patients with rheumatoid arthritis and SLE secrete autoreactive / polyreactive antibodies that can recognize classic autoantigens with low affinity [90]. Under the influence of genetic or environmental factors, these autoreactive / polyreactive mature naive B cells can be differentially activated, resulting in the appearance of B lymphocytes which produce antibodies with high affinity for autoantigens [91]. Breaking self-tolerance can occur not only due to abnormalities of apoptosis (see above), but also due to the modification of autoantigens during an inflammatory, neoplastic or other damaging process [64], because of cross-reactivity between foreign antigens and autoantigens and between antigen epitopes and anti-idiotypes [36]. Several factors (e.g. destruction of tissues and inflammation) contribute to the production of co-stimulatory molecules which participate in the interactions between the immune cells thus leading to a more active autoimmune response, but when the tissues are intact, autoantibody titers remain low [92]. ADs can be also triggered by the external influences of adjuvants, adjuvant-like substances and polyclonal immunostimulants, both of natural (infectious and non-infectious) and anthropogenic origin. This polyetiological additive threshold effect on the individuals predisposed to ADs is known as the “mosaic of autoimmunity” concept [93], and the role of inflammatory autacoids as triggers of the intensification of the autoimmune response is postulated by the “danger hypothesis” [92].

Since autoantibodies can appear long before the clinical manifestations of developing ADs, they can potentially serve as predictive biomarkers for these diseases. Thus, there is some data that antibodies to cyclic citrullinated peptides appear many years prior to the symptoms of rheumatoid arthritis and are almost always absent in healthy individuals [94]. However, these autoantibodies are characterized by significantly higher sensitivity and specificity than ANA, which makes them much more valuable as predictive biomarkers for the laboratory diagnostics [95, 96].

At the same time, although there are some evidence confirming that ANA can also be detected in patients with rheumatological diseases long before the development of the disease [97-99], ANA-positive patients do not necessarily develop ADs in the follow-up, at least those diseases which are generally recognized as autoimmune ones [100-102]. One way or another, but the theory of immunological clearance as the main function of natural autoimmunity has evolved in recent years into the concept of functional autoantibodies and their homeostatic role. It is expounded in a number of the papers cited above [29, 38, 42] and the most recent [103] publications.

However, the authors of these works, as it was before, still see the homeostatic role of autoimmunity only in the possibility of natural self-correction of certain disorders by such autoantibodies (related to the elimination of autoantigens and / or interference in their metabolism). In this interpretation, autoimmunity, albeit “beneficial” in such cases, is still related to the disease. That is, according this view, autoimmunity is more likely not a normal, but a compensatory phenomenon.

The ideological influence of the famous Paul Ehrlich's “horror autotoxicus” [104] can be read between the lines of this advanced works on functional autoantibodies. For many years this postulate prevented the majority of the immunologists not influenced by I.I. Mechnikov’s concepts, from the recognition that autoantibodies can be physiological.

At the same time, there is also a more radical interpretation of the phenomenon of physiological autoimmunity, based on the theory of the autoimmune regulation of cell growth and functions. The formation of this theory goes back to the works of A.A. Bogomolets and L.R. Perelman on the effect of small doses of organ-specific antisera (in the terminology of that time - "cytotoxins") - on target organs. According to the modern interpretation of this theory, autoantibodies act as adaptive bioregulators of cell functions like neurotransmitters or hormones both in health and disease [39, 44, 105].

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That is, physiological autoantibodies represent specific signals addressed not only to superficial, but also to intracellular receptors, including genomic ones. These signals are the part of the network of idiotype-antiidiotypic interactions and target not only immune cells, but also other cell types, taking part in the regulation of the cell growth, gene expression, and renewal of cell populations [36, 39, 44, 105-108].

It was shown in a number of works on the model of endocrinocytes (adrenal cortex, adenohypophysis, thyroid gland) that IgG against tissue-specific antigens of the cell nuclei, represented by the complexes of DNA and non-histone proteins, are able to stimulate hormone biosynthesis in these cells in specific ways and influence their proliferation (which resulted in hyperplasia of targeted organs with prolonged exposure). The effect of cytostimulating IgGs targeting the adrenal cortex was reproduced after hypophysectomy and was able to inhibit atrophy of the adrenal cortex in rats, deprived of pituitary. These antibodies gave a picture similar to ANA in the reaction of indirect immunofluorescence, and serologically identical immunoglobulins were detected in the serum of intact animals by Oochterlony's test [39, 44, 64].

For a long time, the development of this concept was restrained by the prevailing opinion about the inability of antibodies to penetrate into living cells (see above), but the work of the Mexican scientist D. Alarcon-Segovia and Russian authors A.S. Zaichik et al., later confirmed by many other scientists [39, 48-50, 52, 89, 109] demonstrated the ability of ANA (both experimentally obtained and isolated from the sera of SLE patients) – to penetrate into living cells in vitro and in vivo, and showed the biological effects of such immunoglobulins on various genetically determined processes. This concept is also consistent with the data on the enzymatic activity of some antibodies to nuclear antigens in relation to their target antigens (the concept of abzymes) [110].

Thus, an important aspect of the functioning of the system of natural autoantibodies is their involvement as recognizing, signaling or catalytic molecules in the immunoneuroendocrine regulation.

Protective function of autoantibodies in human ADs, especially with regard to natural IgM, is equally important [65]. An inverse correlation was found between the level of IgM autoantibodies and disease activity, severity of lupus nephritis and cardiac manifestations in SLE [111-113], articular lesions in rheumatoid arthritis [75] as well as with the severity of non-rheumatological ADs [70]. It was found that natural IgM autoantibodies are involved in the regulation of IgG reactivity in normal sera by binding and neutralizing them [114]. A similar role is attributed to natural autoantibodies against the IgE receptors in down-regulation of anaphylactic hypersensitivity [38]. It should be noted that pathological processes in immune-dependent diseases are more often mediated by autoantibodies of the IgG isotype, although other isotypes may also contribute [115].

In the light of the the concepts of physiological autoimmunity and natural autoantibodies, it can be expected that not only the upper, but also the lower cut-off levels of some autoantibodies may be diagnostically significant, and protective autoantibodies (including ones against nuclear antigens) will be found to contribute not to the disease, but to the homeostasis. It has been recently shown for anti-DFS-70 autoantibodies in rheumatological ADs [12, 16, 57-59].

In this regard, it is of great importance to establish reference intervals for the levels of autoantibodies, as well as to study the influence of age, sex, ethnicity, and environmental factors - on the occurrence, spectrum and titers of autoantibodies in healthy individuals.

3. ANA: detection, polyspecificity and relation to the ADs pathogenesis

As already mentioned above, a significant proportion of ADs belongs to the scope of rheumatic diseases. ANA are the common biomarker for these disorders. They are found in the sera of more than 90% of patients with systemic ADs, and can also be detected in many other autoimmune, infectious and oncological diseases [5, 116-121]. ANA represent a wide family of autoantibodies of various specificities that bind to nucleic acids and associated nuclear proteins [115, 122-123].

ANF was detected in 1957 in the sera of patients with SLE [122]. Since then, it has become one of the diagnostic criteria for SLE [7, 124, 125], as well as for other systemic ADs with non-organ-specific autoantibodies: systemic scleroderma [14, 120, 121], Sjogren's syndrome, and mixed

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connective tissue disease [7, 23]. ANA also present in rheumatoid arthritis [23], autoimmune hepatitis [126], pernicious anemia associated with primary autoimmune atrophic gastritis [127], Hashimoto's thyroiditis and immune thrombocytopenic purpura [128].

Certain nuclear staining patterns for ANA have been described as clinically significant also in: dermatomyositis, autoimmune myopathies, primary biliary cirrhosis, Crohn's disease, antiphospholipid syndrome, autoimmune cytopenias, and occasionally as paraneoplastic phenomena [16].

Several new associations have been revealed between the presence of ANA and diseases which are not generally considered to be related to autoimmunity: for example, ANA have been found in idiopathic epilepsy [129], ischemic brain disease [130], interstitial lung diseases [131], schizophrenia [132] and other ailments. According to O.V. Danilenko et al. (2020), the level of autoantibodies to double-stranded DNA belonging to the ANA group is increased in various forms of chronic fatigue syndrome (myalgic encephalomyelitis), especially, when the disease manifests after the herpes virus infections [133].

ANF is a historical term introduced in 1960 by the British rheumatologist Eric John Holborow (1918-2009) [134] which was used to characterize the totality of antinuclear antibodies of different specificity, detected by indirect immunofluorescence assay (IFA). IFA is the "gold standard" for ANA detection. Until the mid-1980ies, it was carried out on frozen sections of internal organs of animals or humans (spleen, kidney, liver, etc.), which resulted in additional variability of the test results and hindered interlaboratory comparison. In 1983, a standardized object for ANA-IFA test was proposed [135], namely, HEp-2 cells, which had been cultured by that time for about 30 years in the laboratory as human laryngeal epithelioma strain [136, 137]. The reaction of indirect immunofluorescence with patient sera and fluorescently labeled heterologous antibodies against human immunoglobulins of one or another isotype is carried out according to the standard technique and the result is visualized with the fluorescence microscopy. The serum titer and the fluorescence pattern are assessed [138]. Since 2003-2004 solid-phase ANA testing using multiplex fluorescent immunoassay and similar solid-phase methods began to gain popularity as an alternative to IFA But IFA on HEp-2 cells, which serve as a kind of natural "microplates or even nanoplates" with a set of of autoantigens (circa 100 of them), is still recognized as the most sensitive (and, importantly, visual!) method for ANA detection, despite the improvement of the solid-phase immunoassay methods [16, 138, 139].

When lysates of HEp-2 cells are used as complex antigens in solid-phase immunoassay methods, minor antigenic specificities present in natural cells remain underrepresented, and, therefore, not detected. In addition, antigens are present on cells in the native conformation and among natural microenvironment, while other epitopes can be exposed in solution and on a solid-phase carrier. Therefore, except for cost reduction and increased productivity, solid-phase methods, from our point of view, do not provide any other indisputable advantages over IFA.

The use of standardized HEp-2 cells as a substrate for IFA makes it possible to describe various fluorescence patterns, which reflect the presence of immunoglobulins with different antigenic specificity. Each of the fluorescence patterns (to date, the International consensus on ANA patterns (ICAP) describes 29 such patterns) - is clinically significant for certain AD [16]. Detection of ANA with a description of the type of fluorescence is an important step for the selection of solid-phase immunoassay methods (immunoblotting, enzyme immunoassay, multiplex bead immunoassay etc), which are applied to determine antigen specificity.

However, IFA pattern does not always coincide with the results of the solid-phase assay [140]. In addition, IFA is a more laborious and time-consuming technique in comparison with fully automated biochemical tests, and the visual interpretation of the results could be subjective. Moreover, there are IFA patterns, for which the clinical significance has not been unequivocally established.

Being relevant for the diagnosis and prognosis of mixed connective tissue disease and systemic sclerosis and even criterial for diagnosis of SLE (with sensitivities 90-95%), HEp-2 ANA test is just helpful (with 45-80% sensitivities) for diagnosis of few other autoimmunopathies (autoimmune hepatitis, dermatomyositis/polymyositis, Sjogren's syndrome), but irrelevant in diagnosis of

Hashimoto' s disease or rheumatoid arthritis (due to sensitiivities of 10-20% only) [139]. Therefore, according to a survey conducted among the laboratories, only about 50% ANA tests in the USA and up to 75% in the other countries by 2020 were performed by indirect immunofluorescence [141].

This survey showed that genetically engineered HEp-2000 cells with the overexpression of the SS-A / Ro autoantigen (underrepresented in original HEp-2 cell culture) is becoming more common as a substrate for the determination of ANA staining patterns and are already used by about 24% of the laboratories in the USA , but only about 3% in the other countries. The establishment of automated platforms for reading of the IFA results to a certain degree made it possible to overcome the subjectivity of the method during last decade. The agreement between the results of manual and automated HEp-2 ANA tests reached 92-99%, although the hardships are still great in recognition of mixed patterns, and automated test is combined with manual reading, with still imperfect inter-observer agreement [139]. Hence, there are persisting doubts if ANA revealed by single HEp-2 IFA-test may serve as entry criterion even for SLE, when they present most often, with a recommendation to use combined IFA and solid-phase assay data [142, 143].

In 2020, about 33% of the laboratories used an automated platform for slide preparation, 16% captured images by automated platform, but only 5% used automation for the interpretation of images [141]. Modern consensus guidelines for the interpretation of the results of ANA testing by IFA [16], known as ICAP, developed in 2014-18, have become an important step towards standardization of the interpretation and unification of the nomenclature in this area. Interestingly, that in ICAP workshops, it was agreed that regarding the 29 defined fluorescence patterns the term "disease associations" should be replaced by "clinical relevance".

With the new knowledge and experience gained in the usage of modern modifications of the old ANA test, it became clear that not only the classic term "ANF", but also the other ubiquitous one "ANA test" does not fully reflect the variety of IFA data. The fact is that IFA with HEp2 cells allows to register not only 15 nuclear staining patterns, but also 9 cytoplasmic ones, as well as 5 still other ones associated with the mitosis, where not only chromosomal autoantigens, but also autoantigens related to the cytoskeleton are involved [16]. The most radical proposal was to rename the detection of ANA by IFA to "a test for anticellular autoantibodies" [144]. However, later a more precise name was suggested: "the HEp-2 IFA" [145-146].

Of course, one can recall in this connection the famous: "A rose by any other name would smell as sweet... " - from the mouth of Shakespeare's Juliet [147]. But in this case, the name of the test is an important detail, because different laboratories, reporting to the customer about the test result, interpret the detection of cytoplasmic fluorescence patterns during the ANA test in different ways. Sometimes, for reasons of semantics (after all, it is "test for antinuclear antibodies" that was written in the order!) they report the cytoplasmic pattern of ANA as "ANA negative", although the clinical relevance of the cytoplasmic patterns of fluorescence is beyond doubt, sometimes even for the same diagnoses which are confirmed by nuclear patterns [16, 141 , 145].

4. Detection of ANA in healthy individuals

There is ample evidence in the literature that ANA can be detected in healthy subjects by both IFA and biochemical immunoassay methods [148-153]. Autoantibodies to nuclear proteins are normally present in the sera taken from healthy people and intact animals [154, 155]. Moreover, in healthy individuals, not only autoantibodies to double-stranded DNA were found in blood plasma, but also their anti-idiotypes [156]. Some of the autoantibodies of DNA in the sera of healthy donors are masked by complexes with serum poly-anionic proteins and can be detected after special sample processing, which indicates their wider prevalence in normal conditions than routine laboratory methods show [157]. Therefore, it is very important to distinguish between normal and pathological levels of autoantibodies for the diagnosis of ADs, also when the HEp-2 IFA test is performed.

4.1 HEp-2 IFA cut-off titers

The main disadvantage of the HEp-2 IFA test is its quite low specificity due to the presence of ANA in the sera of healthy donors. In most published sources, including methodological guidelines, only the upper limit of normal ANA titer is indicated, since their complete absence is considered the

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most frequent normal variant (although not the only one!). In view of the above (see Sections 1-2), this approach looks like not sufficient and not up to date.

Autoimmunology is moving towards the recognition of the importance of the normal range of the levels of several autoantibodies, as it has long been accepted for the hormonal and any other bioregulators.

In the literature, different values are mentioned as a cut-off from which the HEp-2 IFA test should be considered positive: 1 / 40-1 / 80 [158], 1/80 [152], 1/100 [117], 1/160 [159, 160], and 1/200 [161, 162]. Therefore, the frequency of ANA-positiveness in different studies will be different depending on the chosen cutoff. According to a laboratory practice survey published in 2020 [141], 50% of laboratories in the world accepted a 1/40 titer as a cutoff.

In a review article by Saikia et al. the importance of the ANA cutoff titer is discussed [163]. The authors have shown that with 1/40 serum dilution, about 20-30% of clinically healthy people had a positive result. When a 1/80 titer was used as a cutoff, this share reduced to 10-12%, when 1/160 and 1/320 titers were used – it falls down to 5% and 3%, respectively. A similar picture is observed when comparing any data of the authors who indicate in their articles the frequency of ANA in different titers (Table 1).

For example, in a Brazilian study of 500 healthy adults, 22.6% of cases were found to be positive for ANA with 1/40 as a diagnostic titer [97]. However, at 1/80, 1/160 and 1/320 dilutions, the rates of positive results were much lower. Other authors from Brazil [164] did not use the 1/40 dilution in their study – therefore ANA positiveness rate among their patients was almost two times lower than in the study by Fernandez et al. (Table 1). Moreover, it was [164] who pointed out the connection between the special pattern of fluorescence associated with autoantibodies targeting DFS-70 antigen and the absence of rheumatological diseases.

Table 1. This is a table. Tables should be placed in the main text near to the first time they are cited.

Author(s), year , [Ref.]	Country(ies)	Age, years	n	Share of ANA positive depending on titers, %					
				1/40	1/80	1/100	1/160	≥1/320	Total
Tan E.M. et al., 1997 [165]	International (USA, Europe, Australia, Canada, Japan)	21-60	125	31.7	1.3	N/A	5.0	3.3	41.3
Fernandez S. et al., 2003 [97]	Brazil	18-60	500	14.6	4.6	N/A	2.0	1,4	22.6
Cacciapaglia F. et al., 2008 [171]	Italy (Filipinos)	25-65	80	N/A	N/A	23.7	N/A	N/A	23.7
	Italy (Italians)	25-69	60	N/A	N/A	8.3	N/A	N/A	8.3
Marin G.G. et al., 2009 [20]	Mexico	12-72	304	35.4	13.4	N/A	3.2	1.6	53.6
Mariz H. et al., 2011 [164]	Brazil	18-66	918	N/A	5.9	N/A	1.0	5,9	12.9
Satoh M. et al., 2012 [21]	USA	20-29	686	N/A	13.1	N/A	N/A	N/A	13.1
		30-39	642	N/A	13.4	N/A	N/A	N/A	13.4
		40-49	581	N/A	11.5	N/A	N/A	N/A	11.5
		50-59	478	N/A	17.4	N/A	N/A	N/A	17.4
		60-69	525	N/A	13.8	N/A	N/A	N/A	13.8
		70 и >	625	N/A	19.2	N/A	N/A	N/A	19.2
Racoubian E. et al., 2016 [179]	Lebanon	<20->70	10814	N/A	N/A	20.0	3.7	2.8	26.5
Morawiec- Szymonik E. et al., 2020 [127]	Poland	18->60	41	N/A	N/A	N/A	N/A	N/A	4.9

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As early as 1997, Tan et al. obtained similar results on the optimal ANA titer cutoff. [165]. They concluded that the titer 1/40 includes almost all patients (high sensitivity), but also a significant part of healthy individuals (low specificity). At the same time, a titer of 1/160 excludes more than 95% of healthy people, but "does not notice" a significant part of patients. Therefore, laboratories must report the results for both titers. Moreover, according to these authors, the detection of low-affinity ANA in small dilutions in persons considered healthy is also biologically and clinically significant. An inspection of laboratories determining ANA, conducted in 2001 in the USA, showed that almost 60% of laboratories use 1/40 as a cutoff titer, 23% use 1/80 and only 14% use a 1/160 dilution [163].

A similar survey was repeated 19 years later on a larger scale, involving not only 942 American laboratories, but also 264 ones from the other countries [141]. It was shown that modern practice in the countries of the Old and New Worlds is very different: While in the USA, 1/40 titer was used as cutoff by 73% of laboratories (that is, more than it was registered 19 years ago), experts from other countries were clearly inclined to a more strict criterion - only 41% relied on the presence of ANA in a titer of 1/40, and the majority of non-American laboratories considered a cut-off titer of 1/80 (44%).

The vast majority of scientific research related to the doctrine of physiological autoimmunity and natural autoantibodies originated in continental Europe (see above), which could influence philosophy and approach of ANA test interpretation by American and European specialists. Apparently, Europe medical community is better prepared to the shift of paradigm towards the existence of physiological autoimmunity.

The authors of one of the papers cited above [163] concluded that each laboratory should determine a regional diagnostic titer for the population, and the authors of the other study [141] reinforced the importance of the reporting to the physicians the titer at which the result is registered, as well as the inadmissibility of labeling the ANA test results as negative if only a non-nuclear pattern of fluorescence is detected.

However, the use of different cut-off levels in the laboratories makes the results inconsistent. For example, parallel testing of 26 samples in two independent laboratories revealed a discrepancy in titers in 18 cases. Since 1/20 and 1/40 titers were used as diagnostic in the first and in the second laboratories respectively, fluorescence at a dilution of 1/20 was not found and therefore reported as negative in the first laboratory. Moreover, the second laboratory regularly tested the sera in dilutions of 1/320 and 1/1280, which also led to differences in the results [148].

Malleson et al. [166-167], based on the results of the analysis of their own data and literature reviews, noted that ANA are detected in healthy individuals (children and adults) up to a titer of 1/320. The authors conclude that if these antibodies are found in low titers (<1/640) and there are no clinical symptoms, laboratory results should be ignored. Gilbrio et al. also believe that ANA are not necessarily associated with AD, and sometimes even in spite of high titers [149]. They concluded that definition of ANA is required only in individuals with clinical signs of ADs.

But if one follows the above logic, then the same ANA titers in the presence of clinical symptoms should be taken into account, and in their absence should be ignored.

The question naturally arises, what to do with atypical, subtle symptoms of ADs?

And what is the meaning and medico-economic justification of a laboratory study if it does not verify a clinically reasonable diagnosis? Such a dual diagnostic interpretation of the test results reduces the significance of these autoantibodies.

This conclusion is confirmed by the results of Abeles et al [151]. According to these authors, the positive predictive value of ANA for the diagnosis of ADs with the 1/160 cut-off titer was 11.6%. Even with a titer of 1/640, the positive predictive value for rheumatic diseases was low - 26.9%, for SLE - 6%, and with a titer of 1/1280 it was 38.9% and 5.6%, respectively.

Similar results were obtained by Turkish researchers [100]. Of the 409 examined children with suspected systemic AD with non-organ-specific autoantibodies who lived in Turkey, 27.6% had positive ANA titers, and only 15% were ultimately diagnosed with ADs. None of the 13 patients with an ANA titer of less than 1/160 had rheumatic diseases. The positive predictive value of ANA was 16% for any systemic AD and 13% for SLE [100].

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Only 1968 (57.3%) of 3432 patients with suspected systemic AD had ANA test positive results at the titer 1/100 or more, and only in 293 (14.9%) from the "positive" 1968 cases the suspected diagnosis was confirmed [101], that is, the applied efficiency of this laboratory test was very low. In a study conducted in Taiwan (China), which involved 355 patients from a rheumatological clinic with positive results of the HEp-2 IFA test, systemic ADs were more common in those with ANA titer of 1/640 or higher than in those who had ANA titers in the range 1/40 - 1/320 [128].

In another study of 205 children with rheumatic diseases, ANA in a titer of 1/20 or more were detected in 67% of cases, but in 494 children with non-rheumatic diseases - in 64% of cases, which completely negates the value of detection of ANA in low titer for rheumatic ADs [167].

The relevance of ANA testing for the diagnosis of those diseases characterized by the rarity of high titers of autoantibodies, for example, for juvenile idiopathic arthritis is especially doubtful, judging by the literature data [168] and our practice [169]. But for the diagnosis of SLE, detection of ANA is more significant, since the disease is usually characterized by higher levels of autoantibodies [169, 170]. However, even for SLE, the diagnostic value of ANA in the studies cited above did not exceed 15% [100, 151].

4.2. Regional, social, and racial-ethnic aspects of ANA prevalence

In different cohorts of healthy individuals, the prevalence of ANA can vary significantly. Thus, Cacciapaglia et al. found that results of the HEp-2 IFA test were positive in 23.7% of healthy Filipinos who migrated to Italy, compared with permanent residents of Italy, in whom the share of ANA-positiveness was 8.3% [171]. Probably, different socio-economic living conditions of these cohorts could be one of the reasons for such discrepancy. Migrants often have a lower socio-economic status than the indigenous population, and this can be the reason for worse health status. In addition, the authors suggested that the prevalence of ANA-positiveness in migrants can be related to the influence of the environment in which they lived before moving. There is evidence that rural residents have a higher incidence of ANA than the urban population, possibly due to exposure to toxic substances used in agriculture [172, 173]. It was shown that 282 (42%) of 668 men living in North Carolina and working with pesticides had ANA in the sera at a dilution of 1/80 or more [174]. The authors of the study concluded that organochlorine compounds could play a role in the increasing level of ANA, and, over time, in the development of ADs.

Satoh et al. reported that the prevalence of ANA in different groups of healthy adults (donors, healthcare workers, healthy volunteers, residents of small towns) varies widely from 1.1% to 20%, depending on the occupation and place of residence [21]. Mexican researchers found that when healthy subjects were tested for ANA, the titers in health care professionals were higher than in healthy donors and even than in the relatives of patients with rheumatological diseases [20]. The health care workers often are enrolled as healthy volunteers giving control sera in comparative studies: for example, according to Tan et al [165], up to two-thirds of the control sera were received from employees of the universities where the laboratories were located, including 13% from healthy individuals who professionally were directly involved in sera testing.

At the same time, there are data from studies (albeit rather old ones), which show that ANA to DNA in healthy volunteers enrolled from the laboratory staff are revealed more frequently than in overall healthy population [175-176].

The geographical features of the ANA prevalence are worth mentioning. because their prevalence varies in different countries. For example, when 557 healthy volunteers from different countries have been tested for ANA (by HEp-2 IFA), the autoantibodies were found in 45% of Colombians, 38% of residents of Kitava Island in Papua New Guinea, 26% of Mexicans, 12% of Italians and Dutch and 11% of Israel residents [150]. According to the Italian authors, the detection rate of ANA by ELISA was 1.3% in a cohort of 149 healthy adults [13]. When the same laboratory method was used to examine 401 healthy residents of Texas (USA), 25% of them have positive results [177]. With HEp-2 IFA test the same tendencies can be noted. Authors of the publications from European countries (where, as mentioned above, laboratories are more likely to take higher dilutions of serum as cutoff) usually report rather low prevalence of ANA in healthy population. For example,

ANA was detected only in 4.9% of 41 healthy residents of Poland (the titer was not indicated) [127]. The level of AHA-positivity among blood donors in the Netherlands at the dilution of more than 1:80 was about 4%, although lower titers were found in 12.7% of cases [178].

The rate of ANA-positivity in the Americas, according to literature, is higher than in Eurasia. Thus, out of 304 healthy Mexicans, 17.9% had ANA at a titer of 1/80 and more, but only 1.3% of these individuals were positive at 1/320 titer [20]. In a study performed in the USA which involved 4,754 healthy individuals over the age of twelve years old, 12.8% of ethnic Mexicans, 13.7% of Caucasians and 15.5% of African Americans were positive for ANA at the titer of 1/80 [21]. In East Beirut (Lebanon), 10,814 healthy individuals were tested, ANA at a titer of 1/100 were detected in 26.4% of cases, and its prevalence over 7 years since 2008 has increased 2.5 times [179]. But in a geographically close region of Turkey, ANA at a titer of 1/100 was detected only in 4 people out of 507 healthy individuals (0.78%) [23]. In studies conducted in the countries of Indochina and the Far East, several different results of ANA prevalence have been reported. For example, among 100 healthy adults from Thailand, only 8 had ANA (1/80 titer) [180]. In an extensive study conducted in Baoding City, Hebei Province, China, just over 5.9% of nearly 20,500 healthy donors were tested positive for ANA, mostly women [181]. Among 33 healthy adults living in Japan, two had ANA in a titer of 1/40, and two more - in titers 1/80 - 1/160 [182]. In the international study cited above, conducted in 15 laboratories (Europe, USA, Canada, Australia, Japan), positive results in healthy adults at a titer of 1/80 or greater were detected in 13.3% of cases, and in a titer equal to or greater than 1/40 - in 31.7% [165].

It is interesting to trace the dynamics of the ANA prevalence in the same country (or population group) for long periods of time. Since this requires referring to biobanks collected over many years according to standard rules, such attempts are rare [179]. A recent research project from the USA which used a biobank, created during the National Survey of Health and Nutrition of Americans, is outstanding because of its scope. Sera of 14,211 US residents over 12 years of age collected over 3 time periods (1988-1991, 1999-2004, and 2011-2012) were routinely tested for ANA in one laboratory by HEp-2 IFA, and the results were correlated with medical history and questionnaires completed by the participants [183]. Findings indicate that there is a clear, statistically significant trend towards an increase in the number of seropositive Americans over the years, especially in the most recent time period (from 11% and 11.5% to 15.5%). ANA is more common in women (20.1%) than in men (11.4%), in those over 50 (20.5%) than in young people (13%) and in African-Americans (18, 1%) - than in other racial groups. It does not show a correlation with body mass index (although over the observation period BMI increased, as did the consumption of alcohol and tobacco products). Contrary to expectations, ANA are somewhat less likely to be present in active smokers (13.1%) than among non-smokers (17.1%), and are more often found among teetotalers (21.3%) than among those who consume alcohol moderately or frequently (14.8%). The recent increase in ANA seropositivity is most pronounced among white men (from 10.2 to 16.4%) and is especially significant among adolescents (from 5% at the turn of the 1980s-1990s - to 12.8% in 2011-12 years!). Discussing these data, the authors vaguely mention the role of factors acting during gestation and in early life.

But we consider, that the most significant change in early life that occurred between 1989 and 2012 in this case is the establishment of more intense national immunization programs and the adjuvant load associated with these programs, as well as increasing intensity of other anthropogenic adjuvant factors. In 1988, in the United States, only 2.9% among 241 healthy children and adolescents aged 4 months to 16 years had ANA at serum dilutions (1 / 10–1 / 40) [184]. And in 2012, the test turned out to be positive already in 11.2% of the tested American adolescents 12-19 years old, although a higher dilution of serum (1/80) was considered as cutoff [21].

There is practically no data on the occurrence of ANA in healthy children and adults living in the largest multinational country of the world – Russian Federation. We detected ANA at a titer of 1/80 or greater - in 22 healthy donors out of 100, in the Urals, in the Sverdlovsk region, of which 5 people had an ANA titer of 1/320, 1 - 1/640 and 1 - 1/1280 [185].

Currently, it is a common knowledge that geographical differences exist not only in the ANA prevalence among healthy individuals, but in the regional distribution in ADs. For example, Lerner et al. [1] reported that in the 21st century the most significant increase in the incidence and prevalence

of AD occurs in the West and North compared to the East and South. This difference is usually associated with a decrease in the incidence of infectious and parasitic diseases in some regions (according to so-called "hygienic hypothesis “). But there is also an impact of vitamin D supply in conditions of different latitudinal sunlight exposure and additional factors associated with urbanization [1, 150].

It can be pointed out that the above information on the ANA frequencies in healthy inhabitants of the New World, in comparison with Europeans, correlates with the data by Roberts et al. [186], who showed, using 52 million medical observation cards from the period 2010-2016, that the multiracial population of the United States also had greater prevalence of ADs, than population of Europe. They identified regional intra-American variations in the prevalence of ADs: SLE was more common in African Americans in the West North Central and South Atlantic regions of the country; multiple sclerosis - in African Americans in the South Atlantic and Pacific regions; rheumatoid arthritis - in Native Americans in the Pacific, West North Central, and Mountain areas. These findings represent one more evidence for the role of the genetic factors in the etiology of ADs.

But there is no clear parallelism between the distribution of the ADs and the regional prevalence of AHA in healthy individuals, apparently due to the incomplete adequacy of interlaboratory comparisons. In addition, due to the migration processes of recent years, the population of European countries is becoming more and more poly-ethnic and multiracial. Therefore, an accurate comparison with geo-epidemiological data 20-30 years ago cannot be done.

Due to the complexity of comparing data from different laboratories, in order to obtain unambiguous conclusions about the geographical and other social and demographic aspects of ANA prevalence, it is preferable to implement large-scale multicenter studies performed according to a single method with the same test systems.

4.3. Ontogenetic aspects of the autoimmunity to the nuclear antigens

Many parameters used in modern laboratory diagnostics have different reference intervals, depending on gender and age. However, methodological guidelines and publications related to ADs diagnosis, often are devoid of any reference to the differences in the normal values for males and females and for people of different age [23, 152, 158, 162]. It is of interest to compare how much the frequency of ANA differs in healthy individuals of different ages, including children. Indeed, there is a general tendency with age to immunosuppression against the background of a chronic systemic excess of pro-inflammatory autacoids, the activation of autoimmune processes in the body and expansion of their spectrum [187]. But there are few studies which describe the ontogenetic dynamics of ANA levels, detected by the same laboratory, although it is methodologically vulnerable to compare the results of different laboratories analyzing this question.

In general, the incidence of ANA in children is comparable to that in adults. However, in the reviewed publications of authors from Europe, Asia, Australia, North and South America, different age groups have been tested and different ANA cutoff titers were used (Table 2).

The geographical features of the ANA prevalence in healthy adults have been discussed above. Probably, the same patterns exist for children and adolescents, which must be taken into account when comparing research results from different countries.

Some of the authors who analyzed the age-related characteristics of the ANA-positivity, concluded that its prevalence increases with age [21, 166, 179]. According to other researchers, no correlation was found between ANA titers and the age of adult donors [20], at least in the range of 20-60 years [165].

Interesting results have been obtained in the study by Fernandez et al. [97]. The authors showed that among 394 adult donors under 40 years old, ANA was detected in 21.1% of cases at a titer of 1/40 and higher, and in 106 adults over 40 years old, positive results were observed in 28.3% of cases. At the same time, when used a cut-off titer 1/320, the opposite results were obtained: the detection rate in the younger group was 1.5%, and in the older one 0.9%. The data on the children and adolescents of different ages are presented in the publication by Hilbrio et al. [149]. Among the surveyed age

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groups: 6 months - 5 years, 5-10 years, 10-15 years and 15-20 years, ANA was most often detected in children from 5 to 10 years old (Table 2).

Table 2. Share of ANA-test positive cases among healthy children and adolescents (N/A – for absence of data)

Author(s), year [Ref.]	Country(ies)	Age, mo/years	n	Share of ANA positive depending on titers, %					
				1/40	1/80	1/160	≥1/320	≥1/640	Total
Arroyave C. et al., 1988 [184]	USA	4 months - 16 years	241	0.4	N/A	N/A	N/A	N/A	0.4
Allen R.C. et al., 1991 [148]	Australia	1-16 years	100	9.0	N/A	7.0	Н.д	2.0	18.0
Hilário M.O. et al., 2004 [149]	Brazil	6 months - 5 years	63	N/A	3.2	1.5	1.5	1.5	8.0
		5-10 years	77	N/A	9.1	5.2	2.6	2.6	19.5
		10-15 years	49	N/A	2.0	4.0	2.0	2.0	10.0
		15-20 years	25	N/A	0.0	0.0	8.0	0.0	8.0
Wananukul S.et al., 2005 [194]	Thailand	7-15 years	207	9,6	2.9	2.9	0.0	0.0	15.4
Satoh M. et al., 2012 [21]	USA	12-19 years	1190	N/A	11.2	N/A	N/A	N/A	11.2
Somers E.C. et al., 2017 [152]	Mexico	9-17 years	114	N/A	5.3	3.5	7.0	N/A	15.8
Attilakos A. et al., 2020 [129]	Greece	4-14 years	40	N/A	N/A	5.0	N/A	N/A	5.0

Presumably, the most common among the young people (30-35 years old) was the anti-DFS70 immunofluorescence pattern of HEp-2 IFA-test, that is suggested to be protective for rheumatic ADs [57, 188]. Finally, a large-scale study from the USA cited above [183] registered a trend towards an increase in the detection of ANA with age, and especially - after 50 years and a flattening of the differences between adolescents and young adults, which were observed among individuals whose samples were taken 30 years ago, but almost disappeared in subsequent periods due to the recently increased frequency of ANA-positiveness in adolescents. It can be concluded that the data of different authors vary, as regards to the influence of the age on the prevalence of ANA-positivity in health. The frequency of the detection of ANA increases with age, but non-linearly.

4.4. ANA IFA patterns in health

The ICAP of 2014-15 describes 28 types of variants of the picture recorded when determining ANA by IFA [188]. Modernized version of the ICAP 2018-19 added to them the 29th pattern, verified by experts [16]. Below is a classification tree of the main patterns denoted by the acronym AC (anti-cell) and numbers (Figure 1) [189].

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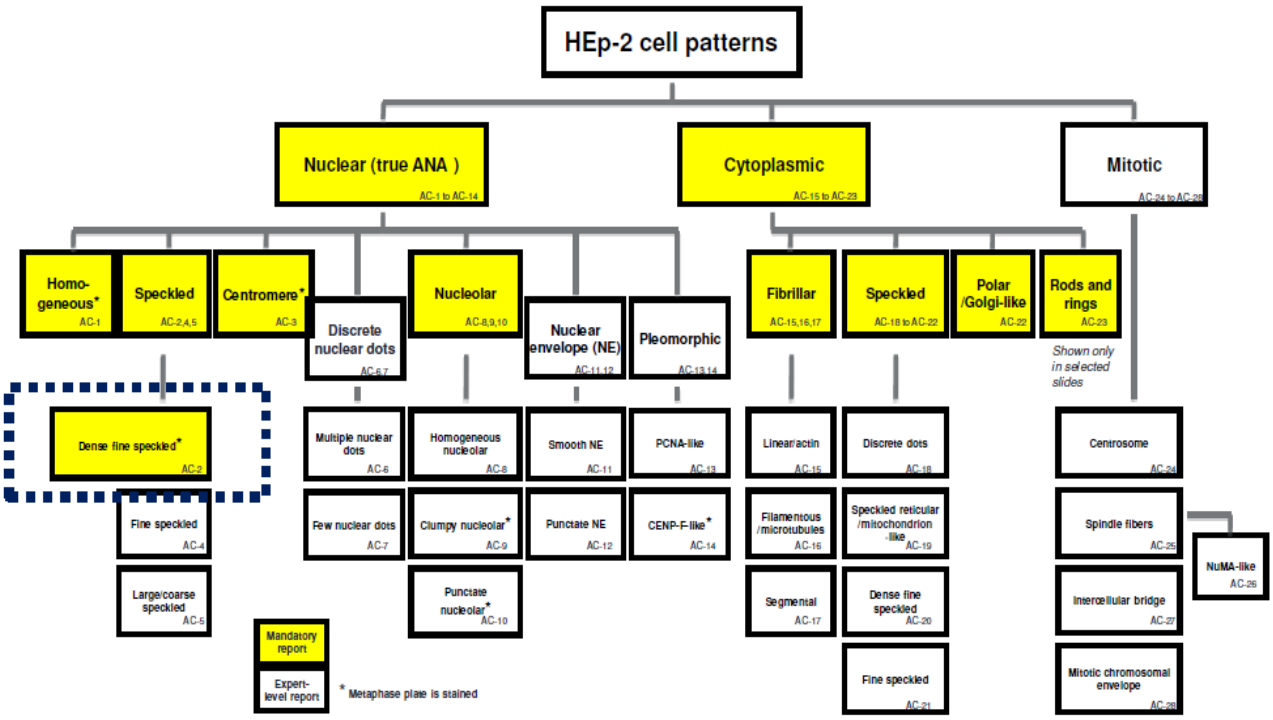

Figure 1. Classification of the main patterns of fluorescence in HEp-2 IFA. On a yellow background, there are patterns mandatory for registration in routine laboratory practice, on a white background – those verified by experts [from: 189].

Among all nuclear patterns observed in HEp-2 IIFA analysis, the main ones and minimally mandatory for the identification by any laboratory during routine analysis are: homogeneous (AC-1), speckled (subtypes which should be reported by "expert-level" laboratories: AC-2, 4 , 5), centromere (AC-3) and nucleolar (expert-level subtypes: AC-8, 9, 10). Among the cytoplasmic patterns, mandatory for routine description are fibrillar (expert-level subtypes: AC-15, 16, 17), speckled (expert-level subtypes: AC-18, 19, 20, 21), polar / Golgi-like (AC -22) and a pattern of rods and rings (AC-23). Mitotic patterns (AC-24 - AC-28) are verified exclusively by the experts.

Homogeneous pattern is characteristic of the presence of antibodies to the main components of nucleosomes: double-strained DNA and histones; it is clinically significant for the diagnosis of SLE, juvenile idiopathic arthritis and chronic autoimmune hepatitis [16, 140, 189-190]. The speckled type of fluorescence is most often observed in a wide range of rheumatological diseases (Sjogren's syndrome, rheumatoid arthritis, juvenile idiopathic arthritis, various forms of lupus erythematosus, systemic sclerosis, dermatomyositis, mixed connective tissue disease, overlap syndromes and undifferentiated connective tissue disease) and is divided into three expert-level sub-types (Fig. 1) [12, 16, 158, 169-170, 190].

With regard to ANA IFA patterns in healthy people, the most interesting pattern is AC-2 – dense fine speckled (DFS) [12, 16] (see below).

ANA to nucleolar antigens are associated with three nucleolar patterns, which are most clinically relevant for systemic scleroderma, but can be also identified in mixed connective tissue disease, Sjogren's syndrome and occasionally – present as a paraneoplastic phenomenon) [16, 140, 158, 189].

A distinct pattern AC-29 has been recently described and associated with antibodies to Scl-70 (DNA-topoisomerase I), which could also cause homogeneous pattern, since the concentration of DNA-topoisomerase is maximal in the nucleoles. AC-29 pattern is highly significant for systemic sclerosis but can be also found in overlap syndrome between this disease and dermatomyositis. [16, 191-192].

Centromere pattern is important for cutaneous forms of scleroderma and CREST syndrome; it can manifest itself long before the manifestation of other symptoms and, together with Raynaud's phenomenon, is prognostically extremely valuable for the early diagnosis [16, 144, 193].

The clinical significance of cytoplasmic and mitotic staining patterns is discussed in detail elsewhere [16, 140, 143].

When ANA is detected in health, speckled pattern is most common, followed by the homogeneous one. For example, Brazilian authors showed that 78% of healthy children positive for ANA had speckled pattern and 11% - a homogeneous one [149]. In an Australian study, when ANA was detected above the cutoff titer in healthy children, speckled, nucleolar, homogeneous, and mixed types of fluorescence were arranged in decreasing order of frequency [148]. In healthy Mexican children, positive for ANA, speckled pattern was found in 50% of cases, homogeneous - in 44%, and nucleolar - in 6% [152]. The results of ANA testing in healthy children in Thailand were somewhat different: when positive results were detected, the homogeneous pattern was determined in 47% of patients, speckled - in 20%, and nucleolar - in 10% [194].

The results of studies involving healthy adults are similar to those obtained in children. Mexican [20] and Brazilian scientists [164] reported that about half of healthy subjects with positive results of ANA test had a speckled type of fluorescence. When examining healthy subjects in the USA, speckled and homogeneous patterns were found in 84.6% of cases [21]. In our study, when positive results of ANA-test were found in healthy Russians, the most common pattern was speckled (more than 60% of positive results), followed in descending order by homogeneous, nucleolar, cytoplasmic and mixed ones [185].

It should be noted that the speckled and homogeneous patterns are the most common ones also in rheumatological ADs [16]. Both these patterns may be due to the presence of antibodies to multiple antigens and are not specific for any particular disease. All this makes the diagnostic process even more difficult because the type of staining pattern cannot be a specific criterion for distinguishing between sick and healthy among the examined persons. Therefore, the reasonable determination of the cutoff for the ANA titer is the most important.

In recent years, special attention has been paid to AC-2 or DFS (dense fine speckled) pattern, which is a subtype of speckled sub-pattern, commonly detected in healthy individuals (especially in young adults) who are positive for ANA [12, 16, 23, 57-59, 101, 164, 188].

This is a fluorescence of interphase nuclei in the form of small dense specks, with a fine-speckled fluorescence of chromosomal regions (but not nucleolar ones) in metaphase cells. It is associated with autoantibodies against the protein DFS70 (see above). The antigen is lens epithelium-derived growth factor or transcription co-activator with a molecular weight of 75 kD.

This transcriptional co-regulator produces an important signal, activating a number of intracellular mechanisms, non-specifically increasing the survival of various cells in response to damaging influences.

AC-2 pattern is rarely found in persons who develop rheumatic AD (0.5–3%), but in healthy people it is present more often (6–11%) [16, 57–59].

Currently, there is evidence that this pattern, when confirming the presence of anti-DFS-70 autoantibodies by immunoassay methods, can serve as a biomarker for the exclusion of systemic rheumatic AD with borderline ANA titers [12, 16, 23, 57-59, 101, 164, 188]. It is possible that these autoantibodies are a characteristic manifestation of "beneficial autoimmunity" (see above) [61]. However, according to the ICAP, they can be considered an “anti-marker” only in the absence of other autoantibodies associated with systemic rheumatic AD [16]. In individuals with the AC-2 pattern it is possible to identify autoantibodies typical for the specific diseases by immunoassay methods and to establish the corresponding diagnoses in about 11-12% of cases [195-196]. Therefore, the presence of the AC-2 pattern is not an “indulgence” of rheumatological health, but a sign that requires clarifying tests by means of other laboratory methods.

Comparing the cytokine profiles and immune cell subtypes of healthy subjects and SLE patients positive for ANA, the American authors found that healthy individuals had significantly lower levels of several endogenous adjuvant-like cytokines (interferons, B-lymphocyte stimulation factor BlyS).

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Both pro-inflammatory IL-12 and stem cell factor c-Ki levels were significantly lower in healthy people, while the levels of the anti-inflammatory IL-1 receptor antagonist - IL-1Ra, on the contrary, turned out to be lower in SLE patients [197]. This corresponds to the danger model and the concept of adjuvant-like effects in defining the line between physiological and pathological autoimmunity (see above).

Thus, for the correct interpretation of ANA test not only the ANA titer, but also the presence or absence of certain qualitative features of the HEp-2 IFA staining pattern, the presence of "protective" anti-DFS70 autoantibodies, and the features of some other parameters of the immune reactivity should be used.

Whether anti-DFS70 autoantibodies, in addition to the properties of an "anti-marker" of systemic autoimmune rheumatologic pathology, represents a biomarker for the presence of any other AD is unclear, although they are often detected in Vogt-Koyanagi-Harada uveomeningitis, which occurs with frequent systemic lesions, in chronic fatigue syndrome / myalgic encephalomyelitis, in atopic dermatitis and interstitial cystitis, less often in Hashimoto's thyroiditis, alopecia areata, sarcoidosis and paraneoplastic phenomena [196, 199]. According to Ochs et al. [199], at least 5-10% of healthy people who do not develop rheumatic diseases during follow-up can be anti-DSF70 carriers.

Since the LEDGFp75 autoantigen, which is the target of these antibodies, is involved in the activation of nonspecific cell responses to damage and coordination of some mechanisms that ensure cell survival, it can be suggested that the generation of anti-DFS70 antibodies may reflect the physiological autoimmunity response to the hyperexpression of LEDGF antigen by the cells exposed to damaging agents - as postulated by the theory of immunological clearance described above [41, 45]. These antibodies can also arise by the idiotype-anti-idiotypic mechanism, in response to the generation of antibodies against pathogenic factors that address the lens epithelial growth factor receptor [36, 60].

In a recent study from Italy, treatment of rheumatoid arthritis with TNFα blocker was associated with the increased expression of anti-DFS70 antibodies, which is an additional argument in favor of their sanogenic rather than pathogenic role [200].

While, from the fundamental standpoint, these autoantibodies are one of the evidences for the existence of physiological autoimmunity, from a clinical point of view they remain a mysterious tile in the ANA mosaic. Recently established antigen knockout DFS70 HEp-2 cells for testing ANA will contribute to the development of the approach to the differential diagnosis between physiologic and pathological autoimmunity. It is also necessary to take into account the epitope differences of the LEDGFp75 domains used in immunoassay methods designed for the detection of these autoantibodies [59, 187, 198]. As for the biological significance of anti-DFS-70 autoantibodies, we cannot judge upon it without direct experimental study of their effect on living cells and laboratory animals.

5. Conclusion

To summarize, ANA can be detected in healthy adults, adolescents, and children. This should be interpreted considering concepts of physiological autoimmunity as well as natural regulatory and functional autoantibodies. Most often in health ANA are found in low titers, which makes it important to revise the cutoff for ANA testing. It is advisable to consider ANA at titers 1/320 or greater as positive in the diagnostic process. It is of great importance to take into account the HEp-2 IFA staining patterns, especially the AC-2 pattern, in view of its role as an anti-marker of rheumatological pathology.

There are geo-epidemiological differences in ANA prevalence among healthy individuals. The reasons may be genetic characteristics of the population, the level of environmental pollution and degree of urbanization, natural and geographical factors, the standards of living and health care in particular areas, however, there is no complete parallelism between the detection of ANA in healthy people and the regional prevalence of AD.

In clinical practice, it is necessary to verify the cutoff for the ANA titer in each particular country or region and rationally combine in practical algorithms both data obtained by IFA and solid-phase assays. First steps to suggest such algorithm were already performed recently [201-203]. Further development of international documents and recommendations covering this area [204] is relevant and should take into account regional and age-related population studies as well as be based on modern achievements in biomedical science and a paradigm shift in the question of the existence of physiologic autoimmunity.

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