Detection of autoantibodies by indirect immunofluorescence and related techniques: the pathologist´s view

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Abstract
The increasing importance of autoimmunity based diseases attracts the interest of pathologists, for whom the wide acceptance of indirect fluorescent antibody (IFA) technique at autoantibody screening is a relevant diagnostic approach. The IFA method has gained perfection by introducing the so called diagnostic mosaics, consisting of various tissue sections (primate and/or mammalian in origin), of HEp-2 cells, of microdots composed of specific purified antigen(s) and/or of transfected HEK cells expressing the target autoantigens. As result of this, the IFA technique dominates not only in the detection of anti-nuclear antibodies (ANA) and of anti-neutrophil cytoplasmic antibodies (ANCA), but is also useful in the search for serological markers in many other autoimmune disorders. Among diseases in question, the inflammatory bowel disease (GAB, PAB, ASCA), celiac disease (ARA, AEmA, AGA) and/or autoimmune hepatitis (AMA, ASMA, ABCA, anti-LKM1, SLA/LP) have emerged as most important. Furthermore, antibodies to gastric parietal cells (APCA), to skin epidermal structures (anti-BP180, anti-Dsg1, anti-Dsg3), antibodies against neurons (anti-Hu, anti-Yo) and glial cells (anti-AGNA) and to several endocrine gland cells (AICA, SCA, OAB, anti-TPO, anti-TG) have recently gained attention. In addition to ANA, the detection of autoantibodies against skeletal or cardiac striated muscles (ASMA/ACMA) is of prognostic value in systemic sclerosis, Sjoegren´s syndrome, dilated cardiomyopathy, myositis and dermatomyositis. At last but not least, detection of anti-sperm antibodies (ASA) has become important due to their association with infertility occurring in the absence of mechanical obstruction of female reproductive system. We bring here an overview of reasonable applications of IFA, which, in combination with additional immunological techniques such as immunoblot (IB), allows a precise identification of several autoantibody target proteins. Though this paper stresses the advantages of IB technique, the enzyme linked immunosorbent assay (ELISA) cannot be neglected, since it has remained inevitable for certain diagnostic decisions (Table 1).

Key words: autoantibody, autoantigen, autoimmune disease, serological diagnostic, indirect immunofluorescence, immunoblot, ELISA.

1. Introduction

For over a half century, fluorescent antibody (FA) staining represents a reliable method of antigen detection (Nairn, 1969). Nowadays, as a rule, monospecific antiserum and/or monoclonal antibody is used to identify the unknown antigen either in organ sections or in cell culture. By the indirect FA (IFA) staining (i.e. applying the specific antibody first followed by the labeled second anti-Ig antibody), not only the antigen can be demonstrated in cells, but vice versa, an unknown antibody present in the tested serum can be identified, when applied to well defined antigen (Goldman, 1968). The IFA staining may occasionally function even when an antibody possibly present in the serum sample, reacts with a less defined antigen complex in properly fixed cells and/or tissues (Coons, 1950). Under such conditions, the reaction may be either positive or negative, depending of the presence or absence of the tested antibody. Based on these considerations, Nožička (1991) claimed that selected human sera may bind to autoantigen(s) present in cryostat sections (coming from different human organs or tissues) and this interaction can be visualized by IFA technique. He also found that nuclei of cells in culture would occasionally react with a proportion of sera originating from patients suffering from certain autoimmune disorders.

The autoantibodies occurring in the sera of such patients may be formed by three different mechanisms. 1. In the case of antigenic mimicry, the foreign (mainly infectious) agent possesses an epitope(s) closely related (or identical) with that present in a human body protein (Fujinami et al., 2006). Foreign antigen may induce undesired immune response when overcoming (braking) the tolerance conferred to the own (body self) antigen(s) (Kamrad and Mitchison, 2001). This relatively simple principle of inducing cross reactive autoantibodes may be true in the case of ACMA (anti-cardiac muscle antibody) production (paragraph 6) and/or AICA (anti-islet cell antibody) formation (paragraph 11.2.). As the anti-nuclear antibody (ANA) induction concerns, genetic defects of early B-cell tolerance may result in such primary Ig repertoire that already possesses substantial nuclear antigen binding capacity by the histocompatibility sequence motifs (or the substrate for such specificities), encoded by appropriately charged complementation determining regions CDR2 and CDR3 (also referred to as hyper variation regions, HVR). Violation of the B-cell tolerance checkpoints in the periphery, namely those described to operate in germinal centers of mice (Shokat and Goodnow, 1995), may further shape the evolving ANA repertoire. This happens either in terms of antibody

affinity and/or of fine specificity for nuclear antigens, or of unexpected cross-reactivity to additional self-antigens as targets (Chang et al., 2003; Simpson et al., 2010). 2. By unmasking the sequestrated body antigen(s) in result of injury or inflammation. Then the continuously acting immune surveillance, mediated by immunocompetent T_h cells and/or mature B cells, gains access to hidden body epitopes. Noteworthy, both mechanisms mentioned here are MHC restricted (Fernando et al., 2008), what explains why only some (but not all) individuals develop autoantibody production. 3. Impaired regulation by T_{reg} cells, which are critical for the maintenance of immunological self-tolerance as well as for immune homeostasis by suppressing aberrant and/or natural, but excessive immune responses (Pan et al., 2009). Due to disruption of the T_{reg} cell activity, which under normal conditions down regulates the increasing immune response (Sakaguchi, 2005), the local activity of the mediator T cells may get prolonged or enhanced. For example, the activity of intestinal TCRγδ+ T cells (which did not undergo to thymus selection) can be suppressed by T_{reg} cells in order to maintain the enteric immune tolerance to own bacterial flora (Park et al., 2010). When overcoming the local tolerance to intestinal flora, the autoimmune inflammatory bowel disease develops.

Nowadays, the IFA method has become a widely accepted and efficient tool for autoantibody detection. Nevertheless, all the alternative methods recommended for testing of autoantibody presence, are essentially similar to those used by immunologists for any antibody testing. The IFA method represents the golden diagnostic standard especially for screening of antinuclear antibodies (ANA) and/or of anti-neutrophil cytoplasmic antibodies (ANCA), for which it functions quite reliably provided that the carefully elaborated international guidelines are exactly followed (Kavanaugh et al., 2000; Rigon et al., 2007; Csernok et al., 2000). In addition to classical HEp-2 cells, genetically modified HEp-2000 cells and/or HEK-293 cells (transfected with autoantigen encoding plasmids) have been introduced in order to create novel specific targets for autoantibody detection. Alternatively, the recombinant antigen coated glass fragments, so called BIOCHIPS, have been introduced for IFA staining. Thus, autoantibodies to several target antigens can be detected just using the fluorescent microscope. The classical, but poorly defined antigens, as found in fixed HEp-2 cells, in polymorphonuclear leukocyte (granulocyte) smears and/or organ sections (from human, primate and/or laboratory mammals), have been supplemented with novel antigen targets, such as such as BIOCHIPS (microdots)

coated with purified proteins and/or transfected HEK-293 cells expressing well defined recombinant proteins. The tissue sections, HEp-2 cells and BIOCHIPS (and/or microdots) are commercially available in preformed combinations, the so called diagnostic mosaics, suitable for individual diagnostic purposes. When the initial screening (at basic serum dilution) is positive, additional more precise identification steps should follow, such as the immunoblot (IB) technique (based on membrane strips carrying specific target autoantigens as purified recombinant proteins) and/or enzyme linked immunosorbent assay (ELISA), in which the recombinant or native proteins are coated to microtiterplates.

Summing up, all methods in which fluorescence microscopy remains a powerful diagnostic approach, are highly appreciated by the pathologist. These, in combination with the IB technique, provide quite satisfactory determination of serological markers in several autoimmune diseases. It should be noted that the detection of autoantibodies by means of recombinant target proteins, which had been coated onto the membrane strips, successfully competes with the traditional ELISA also referred to as enzyme immunoassay (EIA).

Noteworthy, the EIA-based methods of ANA detection are fundamentally different from IFA-based ANA detection methods. The users cannot assume that EIA kits coming from different companies, if used interchangeably, would yield similarly relevant results (Kavanaugh et al., 2000). Despite of these limitations, the ELISA method has remained the most frequently used preferential choice in biochemical and/or immunological laboratories (Keren, 2002), since in principle, it is the most widely accepted antibody detection technique. However, the ELISA results get more value, if recombinant and/or other highly purified proteins are coated to the microtiterplates. No doubt that such novel ELISA may be inevitable even in the pathologist´s laboratory, especially when neither IFA based tests nor IB strips yield a satisfactory diagnostic conclusion (for overview see Table 1).

2. Remarks to ANA diagnostic

For initial screening of anti-nuclear antibody (ANA) as performed by IFA staining, the tested serum can be applied at starting dilution of 1:10. If HEp-2 cells show some of the typical ANA fluorescence patterns (listed in Table 2), the possibly positive serum should be titrated in HEp-2 cells at a series of dilutions (starting from 1:100 to higher ones, for example up to 1:600 or even more) in combination with a standard dilution (as recommended by the

manufacturer) of the FITC labeled anti-human IgG conjugate, which is being applied in the second layer. On the same slide, the basic serum dilution may be checked again in combination with anti-human IgG, anti-human IgA and anti-human IgM conjugates (the latter is not obligatory). As described by others (Ghosh et al., 2007), human serum at dilutions lower than 1:80 may show non-specific staining, resembling to the frequent homogenous and/or speckled patterns as briefly discussed below. Therefore, some authors recommend including the intermediate dilution either of 1:40 or of 1:50, a solution useful in cases of less frequent and/or rare IFA patterns. The basic mosaic used for this purpose at ANA screening consists of HEp-2 cells, sections from primate liver, from rat or mouse stomach and kidney. Especially the positive fluorescence seen in the primate liver section (should be constantly present in the basic mosaic) can be helpful for interpreting the rare and/or questionable ANA patterns (Table 3).

When accepting the principle of positive IFA reading from the serum dilution 1:100, then each sample which had been interpreted as positive at basic dilution 1:10 only, should be examined in IB strips, in order to avoid false negative results. This may be done, for example, using the ANA EUROLINE Profile 3 (antigens nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl, Jo-1, CENP-B, PCNA, dsDNA, nucleosome, histones, ribosomal P-protein and AMA-M2) and/or including the Systemic Sclerosis EUROLINE Profile (antigens Ro-52, PDGFR, Ku, PM-Scl 75, PM-Scl-100, Th/To, NOR-90, fibrillarin, RP 155/RNAP -III, RP 11/RNAP-III, CENP-B, CENP-A and Scl-70). The intensity of positive bands on the strips can be scored by a computer program, the relative scale of which ranges from truly negative (cut-off value 5), through borderline positive (graded 6-10), slightly positive (graded 11-25), definitely positive (graded 26-50) to highly positive (graded >51 by a relative scale). As shown in Table 4, the number of sera scored in our laboratory as definitely ANA positive by IFA during the year 2011, represented only 2.3% of the total examined, while up to 7.4% (3.2 times more) of samples were positive by IB only (these sera were positive at 1:10 dilution when tested by IFA, but were negative at the 1:100 dilution). This finding does not contradict to the statement that IFA is the best approach for ANA screening (Satoh et al., 2007). It just confirms that IB is a more sensitive diagnostic tool for ANA detection than IFA, and that ELISA used for the detection of antibodies against extractable nuclear antigens (ENA) may be efficiently replaced by an alternative line

immunoassay (Peene et al., 2001), for example by Euroline anti-ENA Profile plus 1. As already stated above, an alternative technique should be in any case included into the diagnostic schedule, in order to avoid the false negative interpretation of IFA results (Hoffman et al., 2002). False-negative ANA results as obtained at IFA screening only, would occur by necessity, confirming that IFA technique is useful just for screening, but not for final diagnosis, at least not in clinically suspicious cases of systemic *lupus erythematosus* (SLE) and/or of Sjoegren´s syndrome (Feltkamp, 1966).

The homogeneous nuclear fluorescence is associated with anti-dsDNA autoantibodies, which are often accompanied with antibodies directed against the DNA-binding proteins (such as histones H2/H3/H4) and/or against the whole chromosomal structural subunits called nucleosomes (Bradwell et al., 1995; Verstegen et al., 2009). According to our experience, this relationship is true at least with a probability of 55%. The diffuse homogenous immunofluorescence pattern as seen in HEp-2 cells (Fig.1A) occurs due to the binding of autoantibodies to chromosomal antigens, which are more or less tightly packed into folded chromatin fibers distributed throughout the nucleus (Servais et al., 2009). The anti-dsDNA antibodies alone (i.e. in the absence of antibodies reacting with DNA-binding proteins) can be checked in smears of the haemoflagellate *Crithidia luciliae*, where they react with the kinetoplast structure. If the homogenous pattern shows accentuated nucleoli (Fig.1B), this is a mixed pattern reflecting the presence of additional autoantibodies, especially those reacting with the SS-A, SS-B and/or Ro-52 antigens. Alternatively, the homogeneous pattern may occasionally show relatively faint nuclear fluorescence surrounded by bright perinuclear rim (Fig.1C). The corresponding antibodies then react also with the nuclear envelop proteins such as lamins (Lassoued et al., 1988). In some cases, the nuclear rim fluorescence shows a confluent fine grainy pattern surrounded with a punctate membrane; such finding points at antibodies reacting with the nuclear pore protein(s) rather than with lamins. The intensity of nuclear envelope antigen fluorescence can be increased by staining of such HEp-2 cells, which had been fixed in formaldehyde (Tsiakalou et al., 2006), an option which is not always commercially available.

As shown in Table 4, the most frequent nuclear fluorescence as seen in our laboratory was simply referred to as the speckled pattern (Figs 1D and 1E). The speckled pattern was strongly related (with a probability of 73%) to the presence of

antibodies against the SS-A/Ro-60 kDa (Pollock and Toh, 1999) and SS-B/La-48 kDa antigens, or both. This antibody combination is frequently accompanied with the antibodies to Ro-52 antigen. Sera with SS-A/SS-B/Ro-52 antibodies might show a coarse speckled pattern with accentuated nucleoli (Fig.1D), but as a rule, they reveal the fine speckled pattern (Wiik et al., 2010). The vast majority of SS-B/La-48 antigen positive sera contain antibodies directed towards a linear B-cell epitope spanning the sequence 349–364aa (pep 349–364). Certain findings indicate that a subgroup of SLE patients may possess cross-reacting anti-histone H1 antibodies as well as anti-pep349–364 antibodies, which can be faulty considered as anti-dsDNA reactivity especially by regular ELISA techniques (Touloupi et al., 2005). If antibodies to nucleosomes and/or to dsDNA were found by IB in addition to SSA-A/SS-B antibodies, a mixed fine speckled pattern can be seen in HEp-2 cells (Fig. 1E). The large speckled pattern originally described as nuclear matrix fluorescence can be associated with the nRNP/Sm antigen (Fig.1F). To distinguish these three speckled patterns (fine, large and coarse) needs a great practical experience. We recommend, therefore, to accomplish an IB test to provide precise identification (and/or confirmation) of the target antigen(s). In the absence of IB test result, we prefer the simplified taxonomy which recognizes the common speckled nuclear fluorescence pattern. Attempts to achieve the safe recognition of SS-A/Ro-60 antibodies by means of transfected HEp-2000 cells overexpressing this mentioned antigen (Keech et al., 1996; Polock and Toh, 1999), have not been widely introduced at least not by expected success.

Fig. 2 shows the examples of less frequent and/or rare nuclear fluorescence patterns and their corresponding target antigens as confirmed by IB test. A highly specific pattern is the centromere fluorescence (Fig.2A), which according to our experience, is associated by a probability of at least 65 % with autoantibodies recognizing the centromere nuclear protein B (CENP-B), a kinetochore anchor protein. This pattern may be occasionally associated with the presence of anti-SS-A autoantibody as well; rarely, anti-Jo-1 and anti-Ro-52 antibodies may be found, a combination typical for inflammatory myopathies (Rutjes et al. 1997, see also paragraph 6). In our hands, the next two nucleolar patterns (either homogenous or clumpy, Table 2) occur with a frequency of about 10% out of the total number of sera tested (Table 4).

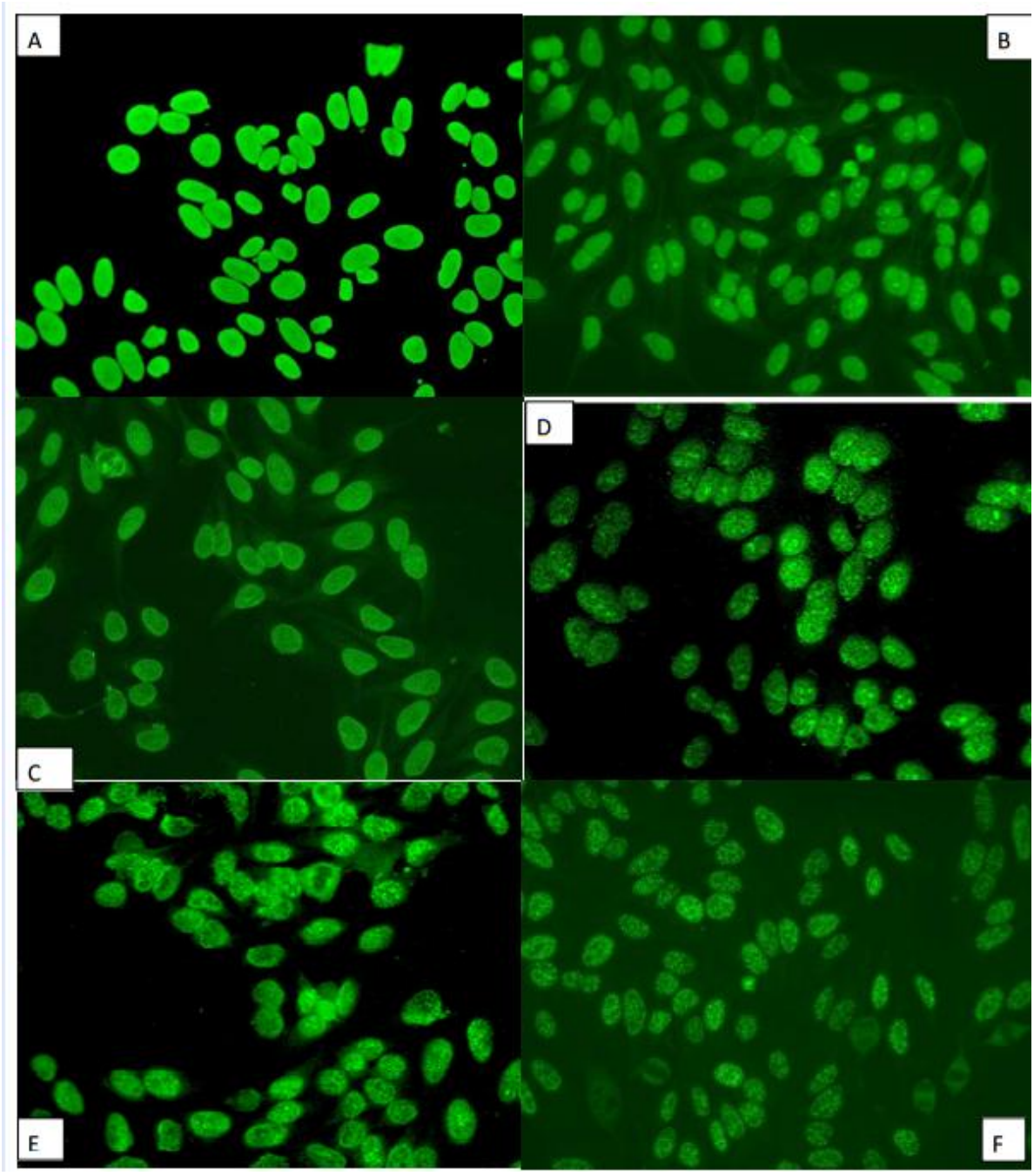

Fig.1. The frequent ANA patterns as seen by IFA technique: A. Homogenous nuclear, no nucleoli seen (IB: antibodies to dsDNA, nucleosomes and to Jo-1 protein were positive, antibodies to PM-Scl slightly positive); B. Homogenous nuclear with accented nucleoli (IB: SS-A, SS-B, Ro-52, nucleosomes, ds-DNA and AMA-M2 positive); C. Nuclear rim positive (IB : negative, i.e. no antibodies found in the EUROLINE ANA Profile 3 strip); D. Coarse speckled nuclear staining with positive nuceloli (IB: SS-A, SS-B and Ro-52 positive); E. Coarse speckled nuclear staining, negative nucleoli (IB: SS-A, SS-B, Ro-52, ds-DNA and nucleosomes positive, histones slightly positive); F. Large speckled nuclear fluorescence (IB: U1-nRNP/Sm antigen positive).

They are associated either with antibodies to PM-Scl antigen (Mahler et al., 2006) or with antibodies to fibrillarin. The latter protein binds to snRNP molecules forming U3-RNP, which is then transported to cytoplasm. Fig.2D shows the lesser frequent nuclear pattern referred to as few nuclear dots. This pattern revealed no positive band in any of the two ANA profile strips mentioned above, but rather reflects antibodies corresponding to a protein called coilin (Andrade et al, 2001). At last but least, an important, though relatively infrequent pattern is the fine grainy (nearly confluent) fluorescence with accentuated nucleoli, mainly related to Scl-70 protein (Fig.2C). Autoantibodies showing this pattern, are important for the diagnosis of systemic sclerosis (namely for its diffuse form). Since this ANA pattern may be difficult to recognize, a corresponding IB test, for example the Euroimmun Systemic sclerosis Prolife strip, seems inevitable for the confirmation of this diagnostic marker (compare Table 1).

The function of target proteins to which the ANA antibodies bind, is briefly summarized in Table 5. This table lists not only the dsDNA (Roslyn et al., 1997) and/or the DNA-binding proteins (histones, nucleosomes), i.e. all the chromosome constituents, but also the small nuclear (sn) RNA-binding proteins such as SS-A, SS-B, Ro52, fibrillarin and others. Some of the latter proteins are involved in sn RNA splicing, another represent enzymes (or coenzymes) related to chromosome duplication (topo-I) and/or enzymes associated with copying the nascent dsDNA strands (for example PCNA, the Proliferating Cell Nuclear Antigen). Other autoantigens are ribosomal proteins and/or translation related enzyme(s), mitochondrial proteins and/or proteins belonging to other cytoplasmic organelles (several, but not all, are listed in Table 5). It comes from this broad autoantigen spectrum that the corresponding fluorescence patterns vary considerably since their localization is either nuclear, or nucleolar or cytoplasmic. Due to repeated but not absolutely successful attempts to introduce a common, single and worldwide accepted international nomenclature (some important examples are shown in Table 2), and contemporarily facing the overall increasing number of the sera examined, introduction of a software based automatic evaluation of basic fluorescence patterns is justified. At their elaboration, different companies created useful patterns which have undergone some degree of unification creating internationally acceptable standards for the reading of IFA results. Nevertheless, alternative immunological tests, either IB or ELISA

are strongly recommended, when aiming to assure a precise diagnosis.

3. Diagnostic of anti-neutrophil cytoplasmic antibodies (ANCA)

ANCA react with various granules which can be found in the cytoplasmic vacuoles of polymorphonuclear leukocytes (Kahlenberg et al., 2001). In the case of cytoplasmic type of ANCA (cANCA) the fluorescing granules are evenly distributed within the cytoplasm of granulocytes regardless to their fixation (Fig.3A). The corresponding autoantibody reacts with proteinase 3 (PR3) as target antigen (Radice and Sinico, 2005). In contrast, the perinuclear ANCA (pANCA) pattern reveals a different distribution of fluorescing granules especially in ethanol fixed smears, where the positive vacuoles accumulate within gaps in between lobular protrusions of nuclei, which seem surrounded by a thicker bright fluorescing membrane (Fig.3B). The localization of fluorescing granules in this case corresponds to the presence of myeloperoxidase (MPO). For exact differentiation of cANCA from pANCA patterns as seen by IFA, a special mosaic can be recommended not only containing the ethanol as well as formalin fixed smears, but also equipped with two BIOCHIPS, each coated with one of the above mentioned enzymes. Noteworthy, the IFA test, which well differentiates cANCA from pANCA, has been declared for more reliable as its ELISA counterpart (Csernok et al., 2000). In addition to coated microdots, an IB strip exhibiting PR3 and MPO antigens is also available. Just recently, the so called anti-PR3-hn-hr-ELISA has been developed, which represents a recombinant inactive antigen (lacking enzyme activity) expressed in human cells along with the native human enzyme extracted from neutrophils (Damoiseaux et al., 2008). When coated to microtiterplates, the above mentioned antigen creates a novel ELISA, which be regarded for equal to the IB and/or BIOCHIP test. In addition to both relatively frequent fluorescence patterns, at least two other but rare ANCA patterns exist, designated xANCA (or atypical ANCA) on one hand, and/or ANA positive ANCA on other hand. The atypical xANCA can be recognized by the negative fluorescence of cytoplasmic granules in formalin fixed granulocyte smears. If the formalin fixed granulocyte smears show faint (borderline or negative) IFA staining, then the acetone fixed HEp-2 cells should be examined. In them, either irregular homogenous staining of some nuclei (ANA positive ANCA or DNA/ANCA) and/or cytoplasmic lysosome-like fluorescence may be noted. The atypical (xANCA) pattern should be further

specified by ELISA, which may prove the presence of antibodies to cathepsin G and/or elastase (Table 6).

It has been hypothesized that the enzymes within the granulocyte vacuoles may become overexpressed if their epigenetic regulation is disturbed. This leads to their increased release into bloodstream and corresponding destruct-tion of capillary endothelium cells especially in kidneys (glomeruli) and lungs (Kamradt and Mitchison, 2001). Table 6 summarizes the clinical significance of ANCA autoantibodies in association with various forms of vasculitis (Geffriaud-Ricouad et al., 1993). In Wegener`s granulomatosis (WG), also designated granulomatosis with poly-angiitis (GPA), the main serologic marker is PR3-cANCA. This disease reveals necrotizing inflammation of small renal arterioles and capillaries (intra renal necrotizing arteritis and focal glomerulonephritis with occasional thrombosis) but no immunoglobulin or complement deposits. At histologic examination of renal biopsy, mononuclear and lymphocyte infiltration as well as giant cell granuloma formation can be seen. The Churg-Strauss syndrome (also called eosinophilic GPA, i.e. EGPA) is characterized by eosinophilic granuloma formation and polyangiitis. Along with the Churg-Strauss syndrome, also microscopic polyangiitis (MPA) is related to the MPO-pANCA marker. The latter disease is more frequent in Japan, in contrast to WG, which more often occurs in Europe and USA (Ozaki 2007).

4. Autoantibodies in gastrointestinal diseases

4.1 Autoimmune liver diseases (hepatitis and related syndromes)

The anti-mitochondrial antibody (AMA) is an easily detectable auto-antibody, which is regarded for typical marker of primary biliary cirrhosis (PBC) (Lindor et. al., 2009). AMA can be found in the cytoplasm of HEp-2 cells and hepatocytes in association with ANA screening (Bogdanos et al., 2003) already at examination of basic mosaic. In addition, it can be seen in renal tubular epithelium cells, since kidney sections are regularly present in the basic mosaic. When AMA has been found at primary screening, then the next step should confirm the exact profile of antibodies present in the serum by including a suitable IB strip (for example the EUROLINE Autoimmune Liver Disease Profile revealing antigens such AMA-M2, M2-3E/BPO, Sp-100, PML, gp210, LKM-1, LC-1, SLA/LP a Ro-52).

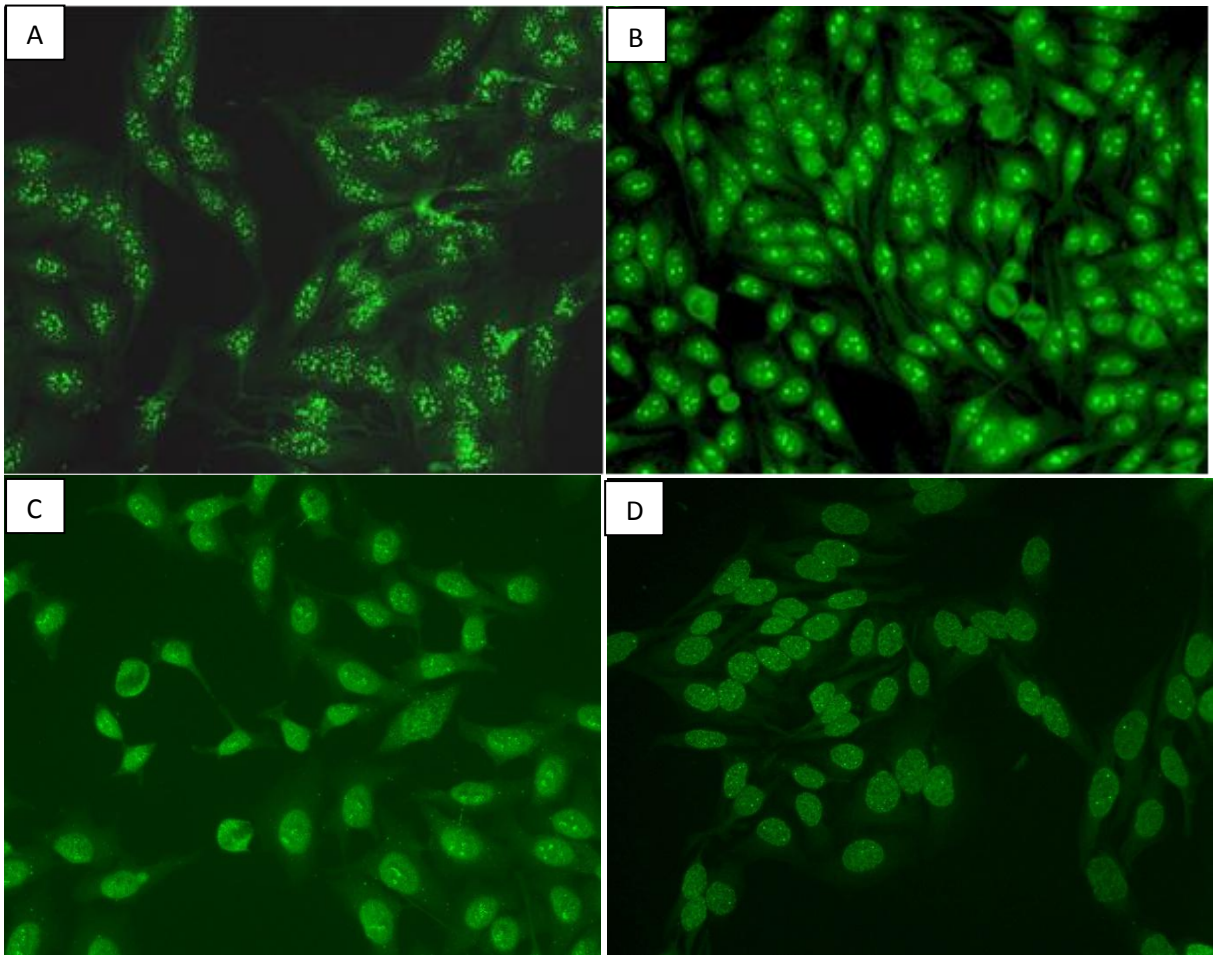

Fig. 2. Less frequent and/or rare nuclear patterns. A. Multiple nuclear dots, centromere pattern (IB: CENP-B positive, SS-A slightly positive). B. Homogenous nucleolar (IB: PM-Scl positive). C. Fine grainy nuclear, Scl-like pattern with the nucleoli accentuated and nucleoplasm homogenous (IB: Scl-70 positive). D. Few nuclear dots (IB: the EUROLINE ANA profile 3 negative; putative antigen is coilin, Adrande et al. 1991).

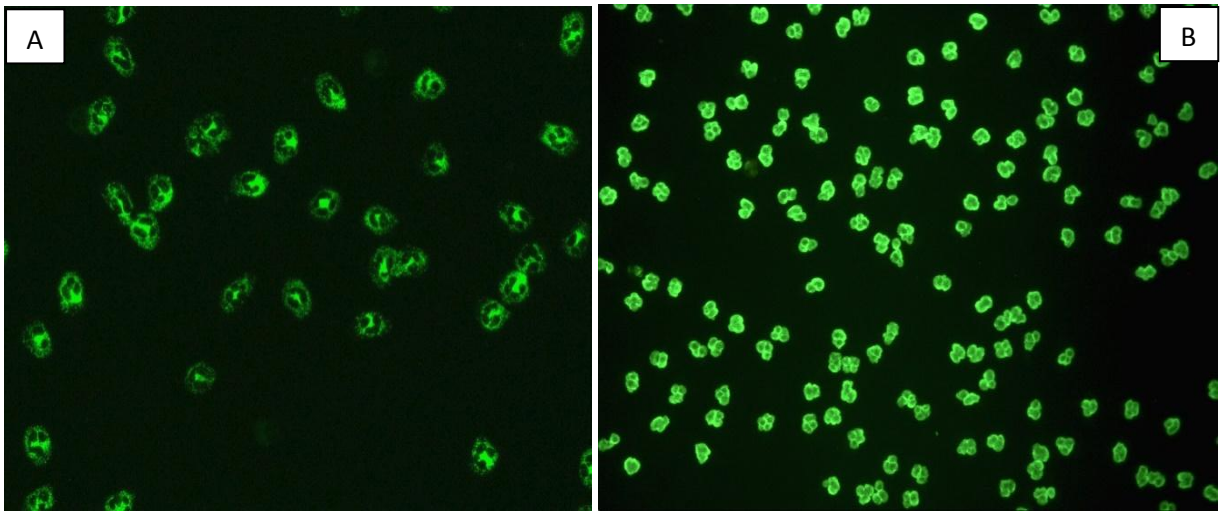

Fig. 3. The two basic ANCA patterns. 3A. Example of cANCA, ethanol fixation, cytoplasmic granules, PR3 positive. 3B. Example of pANCA, nuclear membrane accented with strictly perinuclear localization of granules, formalin fixation MPO positive.

The AMA-M2 (lactate dehydrogenase) antigen in mitochondrial sap is one of several enzymes located inside of mitochondrial crests, out of which M2 is the most frequent auto-antigen. Noteworthy, M2-3E/BPO is a novel recombinant fusion protein consisting of the E2 subunit of alpha-oxo-dehydrogenase, of lactate dehydrogenase and of oxo-glutarate dehydrogenase. While the AMA-M2 and M2-3E/BPO antibodies confirm the diagnosis of PBC, the typical marker for another genuine autoantibody related liver disease, called autoimmune cholangitis (AIC), is the antibiliary channel antibody (ABCA). While in AIC the second marker is pANCA, in PBC the anti-Sp100 (soluble protein100 kDa) occurs as additional marker. As described in HEp-2 cells, antibodies to Sp100 show many fluorescing dots dispersed within the nuclei (up to 20), but their number is still less than in centromere pattern (over 40 dots). The two entities (PBC/AIC) may overlap, and really a corresponding syndrome revealing signs of both diseases had been described (Bogdanos et al., 2008) and referred to as primary sclerotizing cholangiitis (PSC). The PSC is characterized by destruction of small intra hepatic bile ducts followed by fibrosis of the interlobular connective tissue causing liver failure. At the onset of such cases, the liver biopsy shows an extensive lymphocyte infiltration in portobiliary connective tissue (Kaplan and Gershwin, 2005; Lindor et al., 2009).

In children, AIC may combine with the autoimmune hepatitis (AIH), namely with its AIH1 type. AIH1 can be defined as an acute and/or chronic necro-inflammatory liver disorder of unknown etiology characterized by autoimmune features without antecedent viral infections. Thus, AIH1 and AIH2 represent distinct subgroups of auto-immune liver disease, which should be distinguished from each other. Namely, AIH2 may be associated with previous virus infection, especially hepatitis C (Table 7). In AIH1, the anti-smooth muscle antibodies (ASMA) emerged as most important marker. ASMA reacts with the main smooth muscle component actin, but also with vimentin, desmin and other proteins of the smooth muscle micro-tubular system. It is known that actin is a polymeric protein, which also exists in the form of soluble G-actin (Villalta et al. 2008), but what can be detected in AIH1 is the fibrillar F-actin. If using the liver mosaic at primary IFA screening, ASMA will stain the inner and/or outer muscle layer in eosophagus and/or intestine sections, but also the wall of larger arteries

in the liver sections. Furthermore, positive fluorescence may appear in heart muscle sections and in the cytoplasm of HEp-2 cells. It should be mentioned that not only ASMA/actin shows fluorescence in the cytoplasm of the Hep-2 cells, but within their nuclei a homogenous fluorescence pattern occurs as described by ANA. Further markers typical for AIH1 are: positive pANCA as seen in granulocyte smears and the presence of autoantibodies to soluble liver extract (SLA/LP antigen) as detected by ELISA. At last but not least, autoantibodies to the liver hepatocyte membranes can be detected by ELISA (McFarlane et al., 1984; Vergani et al., 2004); the latter namely interact with the asialo-glycoprotein receptor (ASPG-R2).

The basic marker for AIH2 are the antibodies to liver-kidney microsomes (LKM antigen), which should be tested to distinguish the second subtype of AIH. Since originally related to the endoplasmic reticulum of liver hepatocytes, it can be seen already seen at screening, when using the above mentioned liver mosaic, which contains sections from primate as well as rat liver, rat kidney, heart muscle and gastric wall as well as HEp-2 cells. The renal epithelium cells should remain negative (no AMA), both in the cortex as well as in medulla, so that they represent an excellent negative control. In fact LKM is a mixture of several antigens, among which cytochrome P4502D6 (LKM1), cytochrome P4502C9 (LKM2) and uridine-diphosphate (UDP)-glukuronyl-transferase (UGT/LKM3) were identified (Mizutani et al., 2005). Finally, in autoimmune liver disease unrelated to hepatitis C, an autoantibody reactive with liver cytosol (cytoplasm) antigen (LC-1) can be found, which corresponds to formimino-transferase cyclodeaminase (FTCD) as described by Lenzi et al. (1995). In the past, some authors had recognized antibodies to a similar, but less well defined soluble liver antigen (SLA), and/or to a putative liver-pancreas (LP) antigen; these both together were designated SLA/LP (Stechemesser et al., 1993). SLA/LP antibody has been related to drug abuse associated hepatitis D infection; some investigators claimed this AIH category for a separate entity of autoimmune hepatitis (AIH3), in which all the AIH1 markers should be negative (Table 7).

4.2. Celiac disease and atrophic gastritis

Celiac disease is characterized by the presence of HLA-DQ2 and/or HLA-DQ8 haplotypes in the involved patients, by diverse clinical manifestations, a gluten-sensitive enteropathy, and by production of several autoantibodies out of which the anti-endomysium antibody

(AEmA) is the classical one (Chorzelski et al., 1993). Consequently, AEmA has turned out to correspond to the tissue transglutaminase (tTG), respectively to a deamidated gliadin peptide (DGP), both being considered for specific (Table 8). Detection of anti-reticulin antibody (ARA) as described by Calvani et al. (1992), though regarded for useful in basic IFA screening assay, is less specific than AEmA. The sections from gastric and intestinal mucosa show positive AEmA staining in their *lamina propria*, while a fine reticular fluorescence in the subepithelial connective tissue indicates ARA. Only the combined presence of AEmA and ARA predicts the diagnosis of celiac disease with considerably high probability (Mascart-Leone and Lambrechts, 1995; Nadiwanda et al., 2013). There is recommended to confirm the presence of positive AEmA as seen by IFA, by the detection of tissue transglutaminase 2 (tTG2) using appropriate ELISA (Maiuri et al., 2005; Dieterich, 2007). The physiological activity of tTG2 in the gastric and/or intestinal mucosa of patients with celiac disease is enhanced, since this enzyme is activated when cleaving the gluten in ingested food into gliadin. When using the endomysium/gliadin mosaic, the parallel detection of antibodies to gliadin (Ruijner al., 1996) seems of great advantage. Additional components of this mosaic are sections from primate liver, sections from rat oesophagus and/or small intestine along with the BIOCHIPS (microdots coated with a gliadin specific polypeptide). The novel variant of the latter antigen consists of two three times repeated gliadin epitopes (GAF-3x). Since GAF-3x had been coated to microtiter-plates, this test is available also in the form of ELISA (GAF-3x ELISA). In either modification, the gliadin antigen shows high specificity for anti-gliadin antibody (AGA), even though the modified gliadin assay is different from the classical AGA test.

The pathogenesis of celiac disease is a complicated multistep process. Gluten is cleaved by the luminal and brush-border enzymes into amino acids and alpha-gliadin peptides that are resistant to further degradation. It has been hypothesized that the increased intestinal permeability is an early but crucial event supporting the transport of alpha-gliadin through the epithelial barrier by apical-to-basal transcytosis as well as by less well defined paracellular mechanisms (Rubio-Tapia and Murray, 2010). After crossing the epithelial cell barrier and entering the *lamina propria*, the alpha-gliadin peptides are cross-linked and deamidated by tTG2 to produce DGP. The DGP molecules are bound with high affinity to HLA-DQ2/DQ8 molecules expressed on the

surfaces of antigen-presenting cells inducing CD4 T-cell-specific responses in addition to providing help to B cells in eliciting antibody-specific responses. Gliadin is also thought to stimulate the innate immune system directly through the upregulation of IL-15 in intestinal epithelium cells. The cytokine IL-15 is widely recognized to upregulate MIC-A, a stress molecule on enterocytes and the expression of NKG2D receptor of NK cells, promoting an initial enterocyte damage. Taken together, the inflammatory cytokines, apoptotic proteins (granzyme B and perforin), and cytotoxic proteins (metalloproteinases) are thought to be responsible for the early damage to intestinal tissue in patients proven to have celiac disease as seen by biopsy specimens (Kagnoff et al., 2007).

The detection of antibodies to parietal epithelium cells of gastric mucosa glands (APCA) is helpful at distinguishing the type A of atrophic gastritis associated with pernicious anemia from type B closely related to *Helicobacter pylori* infection. In Helicobacter positive patients the degree of their gastric atrophy is directly related to the level of APCA in the serum (Ito et al., 2002). The apical pole of cylindrical parietal epithelium cells lining the surface of gastric mucosa is oriented towards the cavity of elongated crypts. The apical orientation of parietal cells into the lumen direction of crypts is important for hydrochloric acid secretion released to the gastric fluid by sustaining a proton gradient. The corresponding target antigen consists of the both subunits of the special proton pump (H+/K+ – adenosine tri-phosphatase) located in the membrane of parietal cells (Callaghan et al., 1993). Therefore, the fluorescence is strictly confined to surface epithelium of the gastric mucosa crypts. It should be mentioned that the smooth muscle layer of the gastric wall remains negative (no ASMA); furthermore, no positive fluore-scence should be found in kidney tubular epithelium cells (AMA is negative). Since AMA is mimicking the PCA reactivity, in order to avoid heterophilic staining of glandular cells at IFA examination, the forestomach area of rats was discarded in classical test (Hawkins, 1977). When both rat as well as mouse stomach sections were examined in the classical test, the heterophilic staining in the rat sections could be recognized. The non-specific staining could be also avoided by urea pre-treatment before serum application. The novel solution of this problem has been the introduction of monkey stomach tissue for the corresponding diagnostic mosaic.

The parietal cells, scattered in between the pepsin producing glandular

cells, also produce the so called "intrinsic factor", which is a glycoprotein component important for vitamin B12 and Ca^{++} ion resorption. For the detection of intrinsic factor by IFA, the above mentioned monkey cryostat sections are recommended as substrate along with a special BIOCHIP coated with the purified "intrinsic factor" , an antigen isolated from porcine gastric mucosa (EUROPLUS stomach monkey/intrinsic factor mosaic).

4.3. Antibodies related to inflammatory bowel disease (IBD)

Intestinal bowel disease is a common designation for two practically different clinical entities, namely ulcerative colitis (UC) and Crohn´s disease (CD). While the former is characterized by inflammatory lesions located at the surface mucosa of large intestine (especially in *colon sigmoides* and upper rectum areas), in CD slowly developing deep transmural granulomatous inflammation involves mainly the terminal ileum. Unlike to CD, in UC the goblet cell antibody (GAB) is predominantly present. A presumed etiological mechanism of IBD is the dysregulated immunopathologic response to certain antigens of the own bacterial flora (Nakamura et al., 2003). Therefore, in CD heterologous antibodies have been found reacting with epitopes of *Bacteroides caccae* (Ton-B linked outer membrane protein) and *Pseudomonas fluorescens* associated bacterial protein I-2. In UC the ANCA-like antibodies react with H1 histone and the mycobacterial histone 1 homologue called *Hup* B. In general, UC is characterized by the presence of pANCA and/or xANCA reactive antibodies, which are relatively frequent: namely pANCA occurs by probability ranging from 75 to 100 %, while xANCA appears with a probability of 50 %; in contrast, the frequency of cANCA is rare, just at about 10% (Kuna, 2013). Furthermore, in CD the pANCA antibody appears rarely (less than 20%), but more frequently, by at least 40 %, autoantibodies to exocrine pancreatic tissue (acinus cells) can be found (Table 8).

More recently, the chance of correct IFA diagnostic of CD has been improved by introducing the so called CIBD mosaic (Euroimmun), in which the pancreas sections used to detect the pancreas antibodies (PAB), were replaced with HEK cells expressing the recombinant pancreas acinus cell antigens (rPAB/Ag1 and/or rPAB/Ag2). Namely, the mosaic contains two transfected cell lines: HEK-293-CUZD1 expressing the *zona pellucida* (CUB) domain protein (CUZD1) corresponding to the acinus zymogen granule membrane protein and the transfected HEK293-GP2 cell line expressing the pancreas glycoprotein GP2

(Stoecker et al., 2009). Furthermore, the mosaic in question contains the human adenocarcinoma cell line HT29-18N2 producing a goblet cell-like antigen *in vitro*, which is identical with that present in goblet cells of the primate intestinal sections as confirmed using a series of various lectins (Stoecker et al., 1984). It should be noted here that the CIBD associated ANCA is usually formalin as well as MPO negative. Summing up, in UC both pANCA and GAB predominate, while in CD the PAB prevails along with heterologous antibodies to the yeast (*Saccharomyces cerevisae*) surface antigen (ASCA), which occurs with a high frequency of about 90%. The detection of ASCA by IFA is quite reliable, but additional markers, such as heterologous antibodies reacting with several bacterial glycans (ALCA, ACCA, AMCA, anti-L anti-C) and/or above mentioned *Pseudo-monas fluorescence* antigens can be identified by ELISA. Nevertheless, the latter antibodies are not specific for CD, so that in the absence of ASCA their significance seems questionable (Davis et al., 2007).

4.4. The position of serological markers in alimentary autoimmune disorders

The determination of serological markers in autoimmune gastrointestinal disorders seems important for several reasons: 1. Appearance of autoantibodies precedes the development of morphological lesions, which can be detected by intestinal and/or gastric biopsy (Leffler and Kelly, 2006); 2. Since the number of asymptomatic cases prevails, the detection of corresponding auto-antibodies may be helpful for important decisions, such as undertaking of preventive measures (Jennings and Howdle, 2003). 3. Serological markers may be useful at monitoring the effect of therapy and/or for the recognition of relapses, especially in celiac disease. 4. Introduction of novel recombinant protein antigens, either in form of coated BIOCHIPS or expressed in transfected cells, have become a powerful tool for the patho-logist, who prefers evaluating his results in the fluorescence microscope. The serological markers in celiac disease may be easier to examine than the classical duodenal biopsy until now preferred by the pathologist (Rajčáni et al., 2015). The pure and/or recombinant antigens coated to microplates have considerably improved the quality of ELISA.

5. Autoantibodies in renal disease

This paragraph does not encompass the traditional antigen/antibody deposits consisting of immune complexes circulating in plasma. The term anti-mesangial glomerular antibody (AmzGA)

will be discussed from point of view of more precise autoantibody targeting and/or binding, which may concern different glomerular structures. Antibodies to the glomerular basement membrane (anti-GBM) interact with their capillary membrane (Fig.4B), which itself is a complex antigen consisting of the alpha-3 subunit of collagen IV, laminin and proteoglycans. The corresponding anti-alpha-3 collagen antibody is related to a rare clinical entity called Goodpasture`s syndrome (Saxena et al., 1992). In addition to the glomerular capillary membranes, the lung capillary membranes may get involved, so that not only hematuria but also hemoptysis occurs. The so called idiopathic membrane nephropathy (IMN) also designated primary membrane glomerulonephritis (pMGN) is characterized by antibodies to the phospholipase membrane receptor PLA2-R of podocytes, lining the Bowman`s capsule (Schlumberger et al., 2014). To detect the corresponding autoantibody, the recombinant PLA2-R molecule has be purified and linked to microtiterplates (for PLA2-R ELISA). Alternatively, the PLA2-R protein encoding plasmid construct had been inserted in HEK-293 cells. After selection, the transfected cells expressing the PLA2-R antigen can be used to detect the autoantibody in question by IFA. The diagnostic of Wegener`s granulomatosis (GPA) based on the cANCA marker was mentioned above (compare Table 6). In systemic *lupus erythematosus* associated glomerulonephritis, anti-dsDNA antibodies may be present along with certain other ANA, such as anti-Sm and/or anti-SS-A antibodies. In the sanroque mouse model of lupus, pathologic germinal centers (GCs) arise due to increased numbers of follicular helper T (Tfh) cells, resulting in high-affinity anti–dsDNA antibodies that cause end-organ inflammation, such as glomerulonephritis (Simpson et al., 2010). In IgA nephropathy the deposited antigen consists of the fibronectin/IgA complex, which can be visualized by anti-IgA antibody in renal biopsies. As mentioned above, the mesangial deposits of antigen-antibody immune complexes in cases of classical glomerulonephritis are out of the scope of the autoantibody related renal disorders discussed here.

6. Autoantibodies and striated muscle damage

Autoantibodies against striated muscle (ASMA) cause chronic damage, which may be initiated by infection, but in many cases, referred to as idiopathic inflammatory myopathy (IIM), the etiology of is not known (Rayavarapu et al., 2013). The update of the main subtypes of

inflammatory myopathies includes dermatomyositis (DM), poly-myositis (PM), necrotizing auto-immune myositis (NAM) and sporadic inclusion body myositis (sIBM). The fundamental aspects on muscle pathology and the unique pathologic mechanisms of each subset represent diagnostic dilemmas especially concerning the distinction of PM from sIBM and NAM (Dalakas, 2011). DM is a complement-mediated microangiopathy leading to the destruction of capillaries, hypoperfusion and inflammatory cell stress at perifascicular regions. NAM is an increasingly recognized subacute myopathy triggered by statins, viral infections, cancer or autoimmuity with macrophages as the final effector cells causing fiber injury. In PM and sIBM autoantigen specific cytotoxic CD8-positive T cells clonally expand and invade major histo-compatibility-I-expressing (HLA I anti-gen) muscle fibers. Therefore, the define-tion of serological markers in the autoantibody related myositis syndromes is more than welcome (Table 9). For IFA staining, the primate heart muscle/ iliopsoas muscle mosaic is available as such or in combination with many other tissue sections (section array).

An alternative disease of unknown etiology, but with no infectious background is dermatomyositis (DM), which may occur also without skin involvement; the latter is called polymyositis (PM). The skin changes resemble those seen *lupus erythematosus* (LE) and/or in scleroderma. Sharp et al. (1972) described an overlap connective tissue syndrome between local sclera-derma and DM called mixed connective tissue syndrome (MCTD). Since then several investigators confirmed the overlap of clinical picture of myositis with scleroderma skin lesions. Therefore, the serological markers in both diseases are of special importance. On one hand, the Scl-70 and/or coilin antibodies would point at scleroderma (compare Table 2), while MCTD is characterized by high frequency of anti-nRNP/Sm (U1 RNP) antibodies (Sordet et al., 2006). The U1-snRNP complex, but particularly its U1-70 kDa protein component, plays a central role in autoimmune pathogenesis in a subset of systemic LE (SLE) patients as well as in the most of, if not in all, patients with MCTD. Both the U1-RNA component and its specific protein(s), engage immune cells and their receptors in a complex network of interactions that ultimately lead to widespread autoimmune response (Kattah et al., 2010). Another overlap syndrome with the prevalence of PM-Scl antibody (Gutierrez-Ramos et al., 2006) and/or of anti-U3 RNP/fibrillarin antibody may also occur, though the latter would testify muscle involvement (Koenig et al.,

2007). Alternative myositis specific antibodies are those reacting with Jo-1 and other t-RNA synthases (Love et al., 1991). To date, antibodies to additional seven aminoacyl-tRNA synthases (ARS) enzymes were identified, namely PL-7 (threonyl), PL-12 (alanyl), EJ (glycyl), OJ (isoleucyl), KS (asparaginyl), Ha (tyrosyl) and, most recently, also Zo (phenylalanyl). These are present in about 20% of patients with IIM (the frequency of patients with each of non-Jo-1 anti-ARS autoantibodies ranges between 1 and 5%). The antibodies in question mentioned above are found in cases of myositis associated with lung interstitial infiltrate and subsequent fibrosis, the so called anti-synthase syndrome (ASS).

Another form of autoimmune myopathy may be associated antibodies reacting with the signal recognition particle (SRP), a protein complex located at the cytoplasmic surface of the endoplasmic reticulum. Patients with this autoantibody may develop a severe form of idiopathic polymyositis (IPM) with acute onset which can be recognized due to an impaired swallowing. This entity also called necrotizing myopathy (NM), because the muscle fibers are destroyed by a mechanism of antibody mediated cytolysis (Royana-Udomsart et al., 2013). In addition, clinically significant novel autoantibodies such as anti-CADM-140, anti-SAE (small ubiquitin-like modifier activating enzyme), anti-p155 and anti-p140 have been described in the adult and juvenile autoimmuninty based muscle disease (Gunawardena et al. 2009). The anti-CADM-140 antibody is directed against intracytoplasmic MDA-5, the so called melanoma-differentiation asso-ciated gene 5 encoded protein, while the anti-SAE antibody is directed against a post-translational modifier, and the anti-p155/140TIF1 antibodies are against nuclear transcription and cellular differ-entiation factors. If MSAs (myositis specific antibodies) are used to define the clinical syndromes, they may also help to predict outcomes, provided that the results would influence the treatment strategies. In the above discussed cases, the detection of ANA fluorescence patterns may be helpful; staining of a suitable IB strip, either that designed for myositis diagnostic (i.e. Myositis Profile 3: antigens Mi-2, Ku, PM-Scl-100, PM-Scl-75, SRP, Jo-1/PL-7, PL-12, OJ, EJ and Ro-52) or for scleroderma specification (i.e. the Sclerosis Profile: antigens Ro-52, PDGFR, Ku, PM-Scl-75, PM-Scl-100, Th/To, NOR-90, fibrillarin, RP155/RNAP -III), RP11/RNAP-III, CENP-B, CENP-A and Scl-70) detects the antibodies to many protein targets, allowing to specify the presence of corresponding disease markers.

The IIM can be complicated with clinical cardiomyopathy, if the heart muscle becomes widely involved (Van Gelder et al., 2004). In subacute stage of initial inflammation, which is usually of viral origin, an autoimmune destruction of cardiac muscle fibers follows. This then results into fibrosis causing the syndrome of dilated cardiomyopathy (DCM). In typical cases, positive fluorescence of striated muscle fibers (Fig. 4A) can be seen in primate heart muscle sections, while the positive fluorescence of intercalated discs occurs less frequently. In acute phase of myocarditis, a positive fluorescence of sarcolemma can be seen. When the myocyte cytoplasmic organelles show positive fluorescence, then staining of HEp-2 cells is recommended to specify the cytoplasmic ANA pattern (compare Table 2). The etiology of DCM goes back to acute Coxsackie B virus infection, mainly with types 1 (CVB1) or 3 (Fairweather et al., 2005). Experimental CVB3 infection is successful in A/CA and/or A/SW mice, in which the protease activated receptor 2 (PAR-2) considerably impairs immune defense (Weithauser A et al., 2013). In chronic stage, the auto-antibodies react either with the alpha-myosin subunit (the S2 fragment 35 in the atrium muscle as described by Lauer et al., 2000), or with the beta-myosin subunit (in the heart chamber muscles). The destruction of muscle fibers causes troponin release (cellular troponin I, cTnI), which increased serum level is an important prognostic sign (corresponding ELISA is available and recommended for therapy monitoring). Antimyosin auto-antibodies are associated with worse development of left ventricular systolic function and diastolic stiffness in patients with chronic myocarditis. Another marker is the anti-cTnI antibody directed against the polypeptide epitope aa.102-122 of troponin molecule. The interaction of this antibody with the target peptide leads to cytokine release regarded to cause acute heart failure and death. This notion has been confirmed in mice, in which immunization with cTnI caused a severe fibrosis (Kayaa et al., 2010).

Myasthenia gravis (MG) is a peripheral muscle disorder of rather hereditary then inflammatory origin, in which the autoantibodies may react with several components of neuromuscular junctions causing impaired signal transduction (Motomura et al., 2005; Meriggioli and Sanders, 2012). By a frequency of about 30-40% antibodies blocking the nicotine-like receptor for acetylcholine (nAChR) are found, but less frequently, they also interact with the receptor associated muscle tissue specific tyrosine kinase (Gomes et al., 2010). Furthermore, a third component of the

neuromuscular junction complex, the low density lipoprotein receptor related protein 4 (LRP4) may become the autoantibody target. The disease itself is characterized by innervation disturbance of the limb, facial neck and respiratory muscles. As a rule, thymus hyperplasia (or even thymoma) may occur, though some authors deny such association. Most probably, the recognition of self and/or distinguishing the non-self is impaired due to an inherited defect, since T and B cells which are able to mount the immune response against the nAChR molecule can be continuously formed (Klein et al., 2013).

7. Autoantibodies in systemic sclerosis (SSc)

Systemic sclerosis and limited scleroderma are two different forms of the same skin disease; their main feature is the destruction of fibroblasts in dermal connective tissue (the autoimmunity based epidermal lesions will be discussed in the next paragraph). Fibroblast proliferation occurs within the skin as well as below the intima of vessels causing overproduction of collagen resulting into fibrosis. Obliteration of small arterioles develops not only in the skin, but also in kidney, lungs and heart muscle. The proliferative changes within connective tissue are associated with cytokine release, mononuclear and round cell infiltration (Henault et al., 2004). A chronic narrowing process of vessels causes the Raynaud`s syndrome (contraction of the arterioles of hand and fingers accompanied by cramps and pain along with an intermittent interruption of blood circulation). Fibrosis and consecutive scarring develops in the skin, namely at the area of ankles, neck and face as well as of hands. The anti-fibroblast antibodies (AFAs) capable of inducing a pro-inflammatory phenotype in fibroblasts have been detected in the sera of SSc patients along with those which strongly react with topoisomerase 1 (anti-Scl-70 antibodies as described above, Dellavance et al. 2007). The fine grainy Scl-70 pattern as seen by IFA staining of HEp-2 cells (nucleoli accentuated, nucleoplasm homogeneous), is in fact the result of serum antibody interactions with five cellular regions: the nucleus, the nucleolus and the cytoplasm in interphase cells as well as the nucleolar organizing region (NOR) and the chromosomes in mitotic cells. In addition to the anti-Scl-70 antibody, the anti-centromere antibodies (ACA) as well are associated with SSc (Hamaguchi 2010). The presence of anti-Scl-70 antibodies increases the risk for diffuse skin involvement and scleroderma lung disease, whereas centromere antibody pattern often predicts a limited skin involvement in the absence of pulmonary

disease (Dick et al., 2002). A clinical survey of the anti-centromere sera revealed that 67% of them were derived from patients with limited scleroderma. On other hand, the Ro-52 antibodies can be found in patients with a variety of immune connective tissue disorders (Roberts-Thomson, et al. 2003). Anti-nucleolar antibodies define multiple subgroups of patients with SSc. Of these, anti-Th /To antibodies (anti-Th /To) and anti-PM-Scl antibodies (anti-PM-Scl) are associated with limited cutaneous SSc (lSSc), whereas anti-U3RNP antibodies (anti-U3RNP) are associated with diffuse cutaneous SSc (dSSc). However, anti-Th/To autoantibody might be a marker indicating the development of pulmonary hypertension (Khanh and Reveille, 2003). Anti-fibrillarin autoantibodies (which share significant serologic overlap with anti-U3-ribonucleoprotein antibodies) and anti-RNA-polymerase III autoantibodies occur less frequently, similarly as do the anti-Ku antibodies that may be predictive of diffuse skin involvement. Regarding to the complicated evaluation of described markers which contrasts with their important predictive value, a good diagnostic tool is the IB test, for example, the above recommended Systemic Sclerosis Profile strip (paragraph 2 and Table 2).

8. Autoantibodies to skin epidermal structures and related diseases

Autoantibodies directed against the squamous epithelium (including the skin horny spinocellular epithelium) may react with its basement membrane (ABMA) and/or with its intercellular substance (ICE). The best defined related diseases are *pemphigus vulgaris* (PV), *pemphigus foliaceus* (PF) and *bullous pemphigoid* (BP). While the latter (i.e. BP), is a subepidermal blistering disease characterized by massive sloughing off of the whole epidermal layer from dermal connective tissue, then in PF and PV blisters develop due to damaging the supra-basal epidermis (situated deeper from the epidermal surface) or cytolysis occurring nearly in the subcorneal (i.e. upper) squamous cell layer. In BP, the hemidesmosomes representing the adhesion apparatus of basal epidermal cells (providing attachment to the basement membrane) are attacked by autoantibodies, which react with two protein complexes of molecular weight 180 kDa (BP180) and 230 kDa (BP230). The generated antigen/antibody complexes attract complement components and launch an intensive inflammatory response (Jordon et al., 1985). In contrast, autoantibodies in a typical PV react with the intercellular substance, where the target antigens are desmoglein 3 (Dsg3)

and desmoglein 1 (Dsg1) (Lin et al. 1997). Sera containing anti-Dsg3 IgG only, indicate a predominantly mucosal pemphigus with limited skin involvement. Sera containing both, the anti-Dsg3 as well as the anti-Dsg1 are found in the mixed mucocutaneous form of PV, which affects both the skin and mucous membrane (Amagai, 1999). Alternatively, the sera containing anti-Dsg1 only, predict PF, which shows just cutaneous but no mucosal involvement (Table 10). In herpetiform pemphigus (HP), most sera recognize Dsg1, but the rest of them recognize also Dsg3, indicating that HP is a clinical variant either of PF or PV (Hashimoto 2003). When using a dermatology mosaic (contains sections of monkey skin) for IFA staining, ABMA shows granular fluorescence of the basement membrane bound basal squamous cell layer. The positive fluorescence in the intercellular space of squamous epithelium of eosophagus and/or tongue sections would testify the mucocutaneous form of PV with antibodies to both desmogleins. The presence of autoantibodies in question in the serum can be checked using transfected cells expressing either Dsg3 or Dsg1 antigens. It should be mentioned that an alternative to above mentioned transfected cells, namely the Dsg3/Dsg1 antigen specific ELISA, has been available for a relatively long time (Daneshpazhooh et al., 2007). The biochips coated with BP180 antigen are suitable to recognize the corresponding antibody marker in BP.

9. Autoantibodies against neural tissue

When using the neurology mosaic (sections from monkey cerebellum, peripheral nerve, intestinal tissue and HEp-2 cells), the IFA staining reveals at least three basic paraneoplastic antibodies (Geppert et al., 1999; Klotz et al., 2002): 1. Anti-Hu (Fig.4C) which stains the nuclei of Purkynje cells as well as the nuclei of neurons in the *stratum granulosum* of cerebellar cortex, and, in addition, it stains the nuclei of neurons of intramural vegetative ganglia within intestinal wall; 2. Anti-Yo, which stains the cytoplasm of Purkynje cells only, while the granulosa cells remain negative; 3. Anti-Ri, which stains the nuclei of neurons in the CNS (but not in the peripheral vegetative ganglia). In brain sections, the cerebellar and/or cerebral neurons could also interact with GABA decarboxylase (GAD), an enzyme present in the neurons of cerebellar cortex in both, the granulosa and molecular layers (Lancaster et al., 2010). The neurology mosaic, in addition, detects antibodies to myelin sheaths, namely those against the myelin basic protein (MBP), which clinical significance is questionable. The

IB strips yield an opportunity to detect antibodies to several other neuronal antigens, briefly listed in Table 11 (Darnell and Posner, 2006; Dalmau et al., 2008). In some of such rare cases, the IB strips are the exclusive choice, since no other detection method might be available. The IB strips can be especially recommended to demonstrate the antibodies to amphiphysin, PMNA2, GAD65 and several other autoantigens, which importance and clinical relevance has been recently elucidated (Roberts and Darnell, 2004; Lorusso et al., 2004). In addition, a special strip can be used for detection of antibodies against axonal gangliosides such as GM1-3, GD1, GQ1b and others.

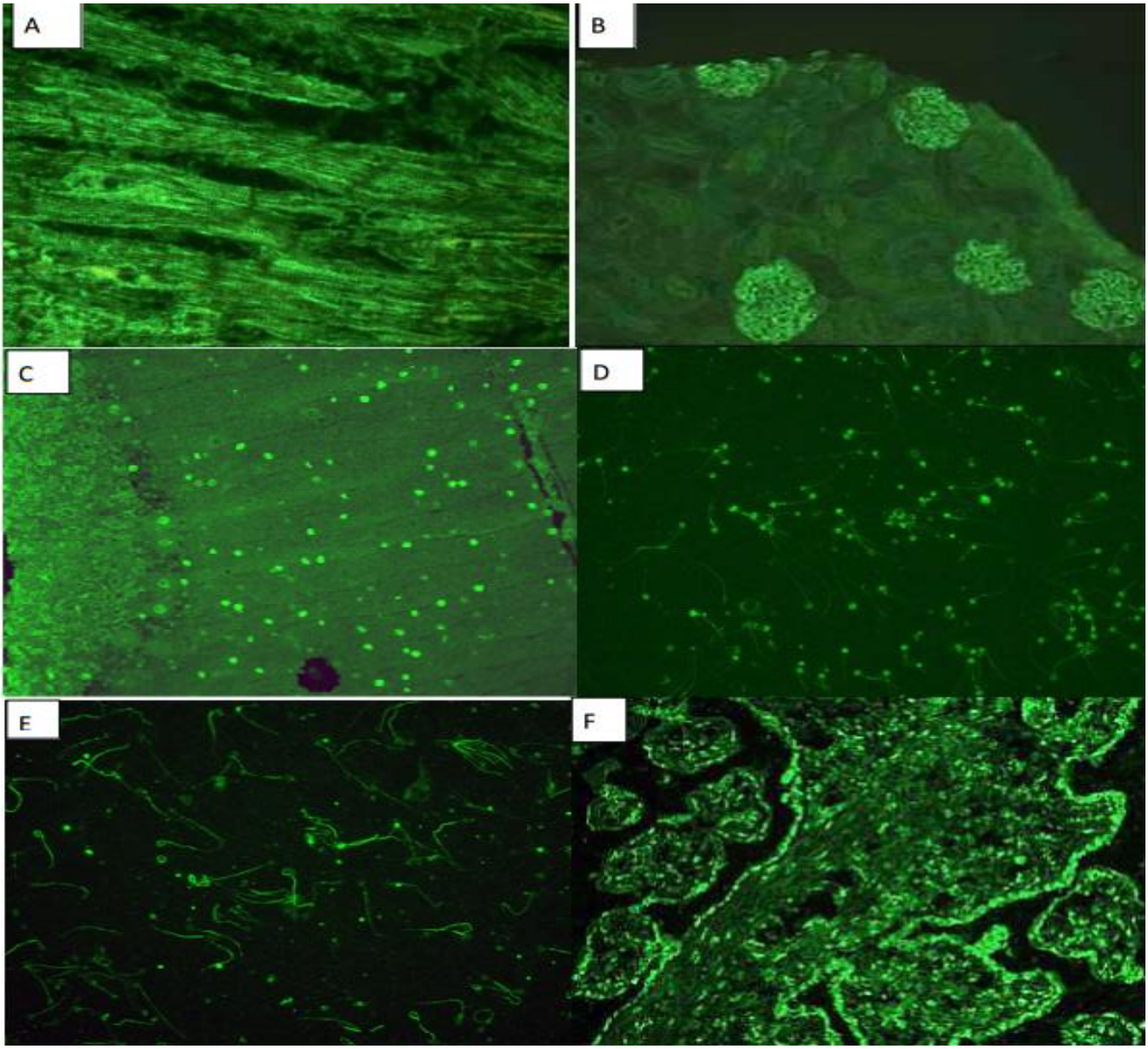

Fig.4. A. Longitudinal section from the primate heart muscle showing positive cross-striations; B. Renal cortex revealing positive glomerular basement membranes (GBM antibody); C. The cerebellar cortex showing positive nuclei of Purkynje cells as well as of neurons within the molecular layer (the layer is negative). D and E: Positive staining of sperm cells; low grade or negative staining of tails, strongly positive heads at the acrosome area (in D); positive tails along with positive head membranes (loop-like appearance, in E). F: Section from placenta showing the villi covered by positively stained syncytiumtrophoblast (anti-SCT antibodies).

10. Autoantibodies and the disorders of reproductive system

10.1. Autoantibodies in infertility

The autoantibody related infertility concerns not only women but also man, especially those antibodies which react with various sperm cell antigens. Thus, the anti-sperm antibodies (ASA) may be present in both sexes. They interact with several antigens of sperm cells, influencing various phases of fertilization (Alberts et al. 2008). The ASA binding site(s) also predict(s) the fluorescence pattern seen in human sperm smears following IFA staining. According to our experience, the fluorescence of whole sperm cells including head and tail (Fig. 4E) can be seen by the frequency of about 40 %, while the fluorescence of the head area only (Fig.4D) occurs by the frequency of about 21 %. In the head of sperm cells, there is occasionally possible to recognize the fluorescence of surface membrane, and/or that of the acrosome vesicle. Frequently, the rear head area (the neck) of sperm cell can be found positive closely at transition to the tail, a finding not regarded for specific. Since the positive scoring of sperm cells in the absence of the tail fluorescence is occasionally obscure, the design of a diagnostic strip which would reveal at least 3 out of the 10 dominant sperm cell antigens is under consideration.

To understand the role of ASA in the fertilization process, some facts should be briefly outlined. During the so called capacitation of sperm cells, occurring in the course of their (tail driven) movement within the oviduct, several surface glycoproteins coming from epididymis are removed, until just the firmly anchored sialoglycoprotein (gp20) of the sperm head surface is exhibited (Focarelli et al., 1998). The gp20 molecule interacts with the *zona pellucida* receptor (ZP2) on the oocyte surface by the help of a cofactor (adsorption protein 50 kDa), which immunogenic peptide has been already identified (YLP12, Naz et al., 2000). During the early events described, the contents of acrosomes is activated (i.e. the acrosome reaction, AR, is launched). The AR occurs due to the activation of a signaling protein called fertilization antigen (FA1), which is anchored within the acrosome membrane. Noteworthy that the acrosome vesicle contains up to 70 proteins from which many are enzymes activated at AR. Here, at least acrosin and the PH-20 protein with hyaluronidase activity should be mentioned (Kim et al. 2008). Both are important at the second (final) phase of sperm cell adsorption. During the AR, several additional enzymes such as lactate dehydrogenase and galactosyl-transferase (gal-T) are released. The latter (gal-T and/or ZP3 protein)

seems essential for the interaction with corresponding oocyte receptor (ZP3R). This interaction (ZP3/ZP3R) initiates membrane fusion, in association with which the fertilin molecule of sperm cell interacts with the integrin molecule on the oocyte surface. Formation of the latter (fertilin/integrin) complex is needed to finalize the sperm cell penetration process. Concomitantly, a conformation change develops not allowing further sperm cells to attach to the oocyte coat. The multistep process outlined above may be blocked at several stages by autoantibodies, especially if they bind to dominant sperm cell antigens, which are inevitable for adsorption and penetration (Suri, 2004). It should be mentioned that impaired (lower) motility of sperm cells may occur due to the binding of autoantibodies to the radial spoke protein located within the tails (Shetty et al., 2007).

We wish to emphasize that the frequency rate of ASA positive sera among the 4.250 samples enrolled to our laboratory during the years 2012/2013 from several districts of Slovakia encompassing an area with nearly 2 million inhabitants, ranged from 3.8 % to 6.6 %. The number of sera tested for ASA represented 22 % of all serum samples examined. The minimum rate of 3.8 % represents definitely positive sera, but does not include those showing faint staining (at serum dilution 1:10) scored as borderline positive. Though several laboratories use to test the sperm bound ASA within the ejaculate omitting the preliminary serum screening step, our work shows that it is reasonable to look for ASA in the blood of both sexual partners, before additional and/or more complicated laboratory tests are performed. The presence of ASA in the plasma of females represents an indicator of its possible local occurrence within the genital tract, even when the autoantibody titer local in local secretions need not correlate with that found in the serum (Stern et al., 1992).

10.2. Autoantibodies against ovarian and placental tissues

Any continuing menstruation disturbance in women represents a good reason for autoantibodies testing, since autoantibodies may be directed not only against sperm cells, but also against the female reproductive organ components, namely the ovarian follicle cells. When looking for the autoimmune background of so called primary ovarian insufficiency (POI) occurring in women whose amenorrhea starts at the age below 40 (LaMarca et al., 2010) by a simple IFA test, the staining of commercially available sections from monkey ovaries is of advantage. In such cases, in addition to the primary follicles, many immature antral

follicles can be seen, which do not differentiate into Graafian follicles, since their thecal cells undergo growth arrest. The connective tissue in between the immature follicles may be infiltrated by lymphocytes. Antibodies react with the *theca folliculi interna* as well as with *theca externa* cells. The target antigens are enzymes which synthesize steroid hormones, namely those belonging to the cytochrome P450 family (i.e. the P450c17/alpha-hydroxylase) or those which cleave the side chains of the steroids, such as P450ssc (compare paragraph 11c). In general, these antibodies are referred to as steroid cell antibodies (SCA). The serum of patients with POI may also react with the interstitial cells, which are derived from *theca externa* and possess receptors for the luteinizing hormone (LH). Taken together, POI means that follicle maturation is delayed and the number of secondary follicles increased. The selective destruction of thecal cells is mediated by specific T cells sparing the granulosa cells so that a syndrome of polycystic ovary develops (Welt et al., 2005). Nevertheless, antibodies to granulosa cells may occur as well, along with antibodies to the ovalbumin present within the follicle fluid. If any anti-thecal antibodies are found by IFA test, the levels of LH as well as of the follicle stimulating hormone (FSH) should be determined in plasma by quantitative ELISA. At least in the beginning, the lower estradiol levels may be compensated by increased FSH production.

Maternal autoantibodies may interact with the surface of syncytio-trophoblast cells (SCT) of placental villi (Fig.4F) causing decidual vasculopathy and thrombosis, which can be seen at histological examination of the placental tissue after stillbirth. The SCT is of fetal origin but does not express the conventional HLA molecules, but a different class designated HLA-G. The maternal antibodies may reach the utero-placental vascular system via the spiralic arteries. The toll-like receptors of SCT cells recognize immunogenic patterns of foreign origin such as lipopolysaccharides in the walls of bacteria during maternal infection. The exact nature of the SCT target autoantigen(s) has not been fully elucidated, though a great deal of work had been done in cell cultures derived choriocarcinomas such as JEG-3 cells; the latter may express antigens reacting with the antibodies coming from mother´s serum. Possibly the best defined autoantigen is the ceramid-beta-decasaccharide, which can be detected also in the wall of fetal erythrocytes by agglutination reaction. Immune complexes formed in the presence of anti-phospholipid antibodies may also reach

the placental vascular system, namely if the mother suffers of autoimmune disorders such as SLE. In the blood of gravid women with SLE several ANA can be detected in addition to anti-phospholipid antibodies, namely anti-dsDNA, anti-SS-A, anti-Ro-52, the angiotensin receptor antibody (AT1-RA), the cardiolipin antibody and others.

11. Autoantibody related endocrine disorders

11.1. Thyroid gland autoantibodies

The autoimmune thyroid disease (AITD) includes has several forms, all of which cause serious functional disturbance of the endocrine thyroid gland function, i.e. hyperthyreodism, hypothyroidism or both. From morphological point of view, there is possible to differentiate the Graves goiter, the Hashimoto thyroiditis as well as post-partum thyroiditis (occurs as complication after delivery by the frequency of 5-6 %) from a thyroiditis developing due to environmental factors. The corresponding autoantibodies, as detected by IFA screening (when using the thyroid mosaic with thyroid gland sections are included), either show positive reaction with the contents of thyroid gland follicles (colloid), or with the epithelial cell lining of the follicle wall, or with both (Gentile et al., 2004). The thyreoglobulin complex (reacts with anti-TG antibody) fills in the follicles; it consists of several components, from which tri-iodo-thyronin (T3) and thyroxin (T4) are especially important. The TG complex is available as an antigen coated to BIOCHIPS, being already included into the thyroid mosaic. If the serum autoantibodies react with the epithelial lining of follicles, the classical fine granular fluorescence of the so called microsomal antigen can be seen in sections, which corresponds to the thyreoid peroxidase (TPO), an enzyme essential for thyroxin synthesis. Anti-TPO is a marker in spumavirus-induced Hashimoto thyroiditis (probability of 75%), but occurs also in Graves goiter and/or in post-delivery thyroiditis. In addition, the autoantibody called long acting thyroid stimulator (LATS) can be present, which interacts with the thyreoid stimulating hormone receptor (TSH-R); LATS block the access of TSH to the follicle cells (Orgiazzi et al., 2000).

11.2. Endocrine pancreas autoantibodes (reacting with Lagerhans islet beta-cells)

Autoantibodies to beta (B)-cells of the Langerhans islets (anti-Islet Cell Antibodies, AICA) can be tested first by IFA using sections of monkey pancreas. The insulin producing B-cells comprise about 70% of total number of endocrine pancreas cells; their granules are equipped with enzymes involved in insulin

synthesis, such as the glumatic acid decarboxylase (corresponding antibody is AGAD), the thyrosine phosphatase (the corresponding antibody interacts with the insulin associated subunit 2, corresponding antibody is AIA2) and the zinc containing cation transporter 8 (corresponding antibody is AZnT8). In addition to these, the main autoantibody target is itself the insulin polypeptide (Winter and Schatz, 2011). While IFA screening shows just granular fluorescence of islet cells, a specification of above mentioned autoantibodies should be made by ELISA, which can be properly designed for each antigen separately using corresponding to microtiterplates (as a rule, the anti-insulin activity is being tested first in a given sample). Noteworthy, AICA production is believed triggered by Coxsackie B virus infection(s), especially by Coxsackie B4 virus. These RNA viruses encode the helper protein (P2C), a viral RNA polymerase cofactor, which immunogenic epitope (aa. 253-265) induces antibodies reacting with a similar epitope of the glutamate-decarboxylase (GAD65) poly-peptide. This is a good example of antigenic mimicry (Fujinami et al., 2006). In children and young adults at the age up to 15 year, Coxsackie B infection may cause juvenile diabetes (type 1), in contrast to diabetes type 2 developing at older age and in seniors. The destruction of B cells in juvenile diabetes may be so extensive that insulin production considerably decreases. The risk of type 1 diabetes is strongly associated with certain HLA antigen types (in Europe mainly DRB/DQB), especially with haplotypes DRB1*0301-DQB1*0201 and/or DRB1* 0401-DQB1*0302 (Rønningen et al., 2001).

11.3. Autoantibodies to adrenal cortex.

In autoimmune form of Addison´s disease there is insufficient production of mineralocorticoids in the *zona glomerulosa* as well as of glucocorticoids in *zona fasciculata* and also of androgens in *zona reticularis.* Using the monkey adrenal gland sections, IFA staining most frequently shows positive fluorescence of *zona glomerulosa* cells, proving the presence of antibodies to adrenal cortex (AACA). These react with enzymes such as 21-hydroxylase synthetizing 11-deoxycorticosteron, but also with above mentioned side chain cleaving enzymes, namely P450c17 and P450c21 (anti-SCA). In the polyglandular autoimmune syndr-ome, in which antibodies are found against ovarian as well as adrenal gland endocrine cells, both anti-SCA and AACA are present, when detected as individual targets by ELISA (Peterson et al. 1997; Betterle and Volpato, 1998). The polyendocrinopathy mosaic recommended

for IFA test consists of 6 BIOCHIPS coated with extracts from each of the antigens in question: thyroid gland, pancreas, adrenal cortex, ovary, stomach parietal cells and testis Leidig cells.

12. Conclusions

Testing for autoantibodies, which has been a long time regarded for the domain of rheumatology and immunology, has recently attracted the interest of other specialists, such as gastroenterologists (inflammatory bowel disease markers, celiac disease markers, markers of autoimmune hepatitis), neurologists, oncologists, endocrinologists and dermatologists. In addition to classical techniques (IFA staining of cells and tissue sections, classical ELISA), which have been at disposal for laboratory diagnostic during last decades, novel methods have been developed which take advantage of highly purified antigens prepared by recombinant technologies. These antigens were coated to microdots (BIOCHIPS), or bound either to microtiterplates (improved ELISA) or to membrane strips (for IB) and/or expressed in transfected cells. The precise diagnostic of autoantibodies to specific targets is of growing impact in diseases such as systemic lupus erythematosus (SLE), systemic sclerosis (SSc), Sjoegren´s syndrome (SS), dermatomyositis (DM), polymyositis (PM), paraneoplastic syndromes, inflammatory bowel disease (IBD), celiac disease, autoimmune heaptitis, pemphigus and/or bullous pemphigoid and in several endocrine disorders (juvenile diabetes, Addison´s disease, thyroiditis and/or goiter). Infertile couples may also profit from the attempt to elucidate the autoimmune nature of their health problem. Since serology is a non-invasive technique and because IFA staining is relatively inexpensive so that its use it for screening of suspicious serum samples may be strongly recommended. For final confirmation of any IFA result, either IB or ELISA (or both) should be performed, to assure the precise nature of antibodies tested.

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Table 1. An overview of autoantibodies (most frequent antigens in use)

IFA test	IB test	ELISA	Autoantibody
Basic mosaic (sections from primate liver, rat kidney and stomach wall, Hep-2 cells); HEp-2 cells only (for titration), *Crithidia luciliae* smear*	**EUROLINE ANA Profiles (several) Anti-ENA profiles (simple or Plus) Euroline systemic sclerosis profile**	**Anti-dsDNA NCX ELISA*, nucleosomes, ssDNA, PM-Scl, anti-ENA profile plus l and/or anti-ENA profile plus 2 (ribosomal P-protein, nRNP/Sm, Sm, SS-A, SS-B, Scl-70, Jo-1, centromeres, each separately); anti-ENA pool, ANA screen 9 and/or 11, anti-ENA SLE profile 2.**	**ANA (ASMA)**
Granulocyte mosaics (various): granulocytes fixed in ethanol as well as in formalin, PR3/MPO/GBM coated microdots in several combinations	**MPO/PR3 and/or MPO/PR3/GBM**	**Anti-PR3-hn-hr-ELISA (recombinant inactive antigen expressed in human cells together with human native enzyme from neutrophils for cANCA)[1] ANCA profile (PR3, MPO, elastase, cathepsin G, BPI, lactoferrin searatelly) pANCA/MPO**	**ANCA**
Monkey kidney sections, anti-PLA2R IFA in transfected HEK-293 cells, GBM coated BIOCHIP**	**Not available**	**Anti-PLA2R ELISA**, GBM ELISA,**	**AmzgA, anti-GBM, antibody to Bowman´s membrane**
PCA mosaic: monkey stomach wall sections, intrinsic factor coated BIOCHIP**	**Not available**	**Anti-stomach wall intrinsic factor ELISA**, PCA ELISA, intrinsic factor ELISA**	**APCA**
liver mosaic (sections from primate liver, rat oesophagus and gut wall) gliadin coated BIOCHIP (GAF-3x)**	**Not available**	**Anti-tissue transglutaminase ELISA; anti-gliadin (GAF-3x) ELISA****	**AEmA; ARA; AGA**
CIBD mosaic: ethanol fixed granulocytes; *Saccharomyces cerevisiae* smears; intestinal goblet cells in culture (HT-29-18N2), recombinant pancreas antigens (rPAg1/CUZD1 and rPAg2/GP2) expressed in transfected cells	**Not available**	**Glycan antibody ELISA : ALCA, ACCA, AMCA, anti-L, anti-C; Anti-*Pseudomonas* ELISA: OmpC; Cbir/flagelin; anti-I2 antibody; Glycosyl-phosphatidyl inosin (GPI) ELISA**	**Markers in UC versus Crohn´s disease (for differential diagnosis, compare Table 8)**
Hepatitis mosaic: sections from primate liver, rat kidney, stomach wall; HEp-2 cells; actin expressing cells (VSM47)	**liver profile (LKM1, LC1, SLA/LP, AMA-M2); Liver disease profiles 14 antigens (either for PBC or for AIH),**	**SLA/LP ELISA Anti-ME2-3E/BPO ELISA (branched chain alpha-keto-acid dehydrogenase / acetyltransferase of pyruvate dehydrogenase /succinyl transferase of oxoglutarate dehydrogenase**	**ASMA, LKM, AMA in AIH versus PBC/IBC diagnostic (compare Table 7)**
Dermatology mosaic: oesophagus, salt split skin, gall bladder mucosa; BP180 antigen coated BIOCHIP; cells transfected with plasmids encoding desmoglein 1/3, collagen type VII NC1	**Not available**	**ELISA: for PB230-CF antigen; for desmoglein 1and 3 antigens; envoplakin and/or collagen VII antigens, BP180 antigen ELISA**	**ABMA/ICS**
Striated muscle mosaic: sections from primate heart and rat iliopsoas muscles	**Myositis profile(s), IIM profile**	**Anti-CDM-140 ELISA; anti-SAE ELISA; anti-TIF1gamma ELISA; anti-alpha-myosin ELISA**	**ASMA/ACMA**

* Table 1 continues

Monkey pancreas sections (Langerhans islands should be present)	**Not available**	**Anti-GAD/IA2 ELISA**	**AICA**
Thyroid gland sections, TG (thyreoglobulin) coated BIOCHIP	**TPO/TG strip**	**TSH ELISA, TSHR /TRAb ELISA; TPO/TG ELISA**	**AITD**
Not available	**Not available**	**Anti-CCP (cyclic citrullinated peptide) ELISA, rheumatoid factor (IgM) ELISA**	**RA (not autoantibody related)**
Neurology mosaics: sections from monkey cerebellum, peripheral nerve, intestine and pancreas, rat hippocampus, transfected HEK cells expressing AQP4, NMDR, AMPAR1/AMPAR2, GABABR, LGI1	**Neuronal antigens profiles, paraneoplastic antigen profiles, Ganglioside profiles, EUROLINE anti-SOX1**	**NMDR ELISA**	**Anti-Hu; anti-Yo; anti-Ri; rare syndromes: anti-NMDR; anti-GABA-R and others, compare Table 10**

***for dsDNA antibody detection; ** equal tests; [1]Damoiseaux et al., 2008;**

Abbreviations: ANA = anti-nuclear autoantibody; ANCA = anti-neutrophil cytoplasmic antibodies; AmzgA = anti-mesangium autoantibody; APCA = anti-parietal cell autoantibody; PR3 = proteinase 3; MPO = myeloperoxidase; GBM = glomerular basement membrane; PLA2R = phospholipase A2 receptor; ASMA = anti-smooth muscle antibody; LKM = liver kindey microsome (autoantibody); AMA = anti-mitochondrial antibody; UC = ulcerative colitis; ABMA/ICA = anti-basal membrane antibody/; ABMA/ICS = anti-basement membrane antibody/intercellular substance; AIH = autoimmune hepatitis; ASCMA = anti-striated/cardiac muscle antibody; AICA = anti-islet cell antibody; AITD = autoimmune thyroid disease; PBC = primary biliary cirrhosis; SLA/CL = soluble liver antigen/liver cytosol; RA = rheumatoid arthritis, TSH = thyroid stimulating hormone; TSHR = thyroid stimulating hormone receptor. For more explanations see legend to Tables 6, 7, 8 and 9.

Table 2 The ANA fluorescence patterns in HEp-2 cells: comparison of nomenclature, target antigens and of related diseases

CANTOR Taxonomy[1]	EULAR taxonomy[2]	EASI recommendation[3]	Related antigen(s)	Associated disease	Simplified pattern[4]	Frequency
Nuclear membrane						
Membrane patterns (smooth or punctate)	1.1.Smooth membraneous 1.2. Punctate membraneous	Nuclear rim or envelope	lamin, gp210 porin (p62)	SLE, RA, PBC, IM, CAH	Nuclear rim (membrane)	rare
Nuclear patterns						
homogeneous	2.1.Homogenous nuclear	Homogenous	dsDNA, histones, nucleosomes	SLE, vasculitis, JIA	Homogenous	Frequent (24 %)[5]
Speckled: large coarse fine	2.2.1. Large speckled 2.2.2. Coarse speckled 2.2.3. Fine speckled 2.2.4. Fine grainy (Scl-like)	 Coarse speckled Fine speckled	 U1-snRNP, Sm SS-A, SS-B, Ro-52, Scl-70 (topo-I)	 SLE, MTCD, SSc, SS, Raynaud´s sy, UCTD SS, SLE, IM, MCTD	Speckled (granular)	Frequent (44%)[5]
Centromere	2.2.6. Centromere pattern	Centromere	CENP-B, -A (kinetochore)	limited SSc, Raynaud´s sy	Multiple dots	Frequent
Multiple dots	2.2.7. Multiple dots 2.2.8. Coiled body pattern	Multiple dots Few nuclear dots	Sp100, PML bodies, coilin, RNA polymerase III	PBC, SS, CAH	 few dots	 Rare
Pleomorphic speckled	2.2.5. Pleomorphic pattern	Pleomorphic (cell cycle related)	PCNA (proliferating cell nuclear antigen)	SLE, SS,		rare
		Dense fine speckled[6]	DFS70/LEDGF-75	Lymphoproliferation, inflammation, none		rare
Nucleolar pattern(s)						
Homogenous	3.1. Homogenous	Nucleolar fluorescence	U3-snRNP (fibrillarin) PM-Scl, To/Th	SSc, IM	Nucleolar Fluorescence	Less frequent (10%)[5]
Clumpy	3.2. Clumpy					
Punctate	3.3. Punctate					
Mitotic (spindle) apparatus (MSA)						
Centriole/centrosome	4.1. Centriole pattern	Centriole/centrosome (spindle apparatus)	enolase, ninein, pericentrin	SSc, Raunaud´s sy, Inflammation	Spindle apparatus	Less frequent
Spindle fiber	4.3. Spindle fiber					
Spindle pole (NuMA)	4.2. Spindle pole	MSA-1, -2, -3 (mitotic spindle apparatus)	centrophilin	RA, inflammation (mycoplasma, pneumonia)	Spindle pole and fibers	Less frequent
Midbody (MSA-2)	4.4. Midbody					
CENP-F (MSA-3)	4.4. CENP-F (MSA-3)					
Cytoplasmic patterns						
Diffuse	5.2. Diffuse cytoplasmic	Discrete speckled	Early endosome antigen 1, processing bodies, lysosomes, multivesicular bodies	SS, SLE, PBC, RA, UCTD, neurological complications (conditions)	Cytoplasmic fine granular	Less frequent
Fine granular (speckled)	5.1. Fine speckled Cytoplasmic					
Mitochondrial	5.3 Mitochondrial like					
Lysosomal	5.4. Lysosomal like					

* Table 2 continues

Golgi-like	**5.5. Golgi like**	**Golgi-like**	**Golgi proteins/golgins**	**SS, SLE, RA, overlap syndrome,**	**(small) vesicular**	**rare**
Actin-like	**5.6. Actin-like**	**Cytoplasmic fibers**	**Actin, vimentin, cytokeratin, tropomyosin**	**DM, CAH, inflammation**	**Cytoplasmic fibrillar**	**Less frequent**
Vimentin-like	**5.7. Vimentin-like**					

[1] Wiik AS et. al. 2010; [2] European League for Rheumatism 2006; [3] Agmon-Levin N et 33 coauthors, 2013;
[4]Based on a simplified ANA fluorescence pattern as recommended for digital (computer) reading (Rigon et al., 2007; Kivity et al., 2012) [5] according to the author´s statistics from 2011: out of the total 5518 serum samples examined for ANA, only 127 showed the one of five basic patterns at dilution 1:100 (compare Table 4). [6] Mahler M et al., 2012;
Abbreviations: EULAR = European League for Autoimmunity and Rheumatism; EASI = European autoimmunity standardization initiative; SLE = systemic lupus erythematosus, RA = rheumatic arthritis, SSc = systemic sclerosis, DM = dermatomyositis, MTCD = mixed connective tissue disease, IM = inflammatory myopathy; NuMA = nuclear mitotic apparatus; PBC = primary biliary cirrhosis, UCTD =undifferentiated connective tissue disease, SS = Sjoegren ´s syndrome; CENP = centromere protein, JIA = juvenile idiopathic hepatitis; CAH = chronic autoimmune hepatitis.

Table 3. IFA staining in liver sections as compared to the Hep-2 cell patterns in ANA screening

Hep-2 pattern	Liver pattern	Corresponding target antigen (determination of the IB profile recommended)
Nuclear fluoresecence		
Nuclear membrane	**Nuclear membrane (hepatocytes)**	**Lamins (A, B, C, Gp210)**
Large (or coarse) speckled	**Bright (speckled) nuclear (hepatocytes)**	**U1- nRNP (nRNP/Sm)**
Fine granular (Ku)*	**Patchy clumpy nuclear (hepatocytes)**	**EUROLINE Mi-2/Ku profile**
Multiple dots (<20)**	**Nuclear dots (hepatocytes)**	**Sp100, PML bodies**
Confluent fine granular***	**Fine granular (hepatocytes)**	**Scl-70**
Nucleolar (homogenous)	**Nucleoli positive (hepatocytes)**	**U3-nRNP/fibrillarin; PM-Scl**
Homogenous (diffuse IF)	**Homogenous nuclei (hepatocytes)**	**dsDNA (kinetoplasts in *Crithidia lucilae* smears);**
Cytoplasmic fluorescence		
Fine granular (diffuse)	**Faint cytoplasmic (hepatocytes)******	**Jo-1, ribosomal P-protein******
Lysosomal (multivesicular)	**Coarse granular (hepatocytes)**	**Lysosomal proteins (antigens)**
Mitochondrial	**Granular cytoplasmic (nuclei negative)**	**Mitochondrial M2, M3, M5 and M9**
Fibrillar (actin)	**Small bile canaliculi**	**F-actin**
Fibrillar (vimentin)	**Nonspecific (negative)**	**Vimentin**
Golgi-like cytoplasmic	**Granular cytoplasmic (paranuclear)**	**Golgins**

* **may be similar to the very frequent fine speckled pattern seen by SS-A/SS-B autoantibodies (liver negative)**

** **differs from centromere dots (>40)**

***nearly homogenous, nucleoli accentuated**

******the hepatocyte surface membrane may show accentuated fluorescence**

Table 4. Autoantibodies scored for positive by IB test, but not by IFA method*

Group A	Group B	Target antigen(s)
204/407 (50 %)	56/156 (36 %)	Autoantibodies to SS-A, SS-B and Ro-52 only or in combination with additional antigens
60/407 (14.7 %)	19/156 (12.1 %)	Antibodies to dsDNA and chromosome-related antigens (nucleosomes and/or histones) only or in combination with additional antigens
53/407 (13 %)	14/156 (8.9 %)	Autoantibodies to Scl-70 only, or in combination with additional antigens
91/407 (22.3 %)	67/156 (42.3%)	The rest of IB strips** with autoantigens PCNA, Jo-1, nRNP/Sm, Sm, CENP-B, Rib. P-protein, PM-Scl, AMA-M2
Total 407	Total 153	

***according to the author´s data, the positive fluorescence seen at basic dilution (1:10) only (but not regarded for positive by IFA staining) was found in 407 samples out of 5 516 sera tested (7.4 %, group A) and/or in 153 out of 2.615 samples tested (5.9 %, group B). In the group of sera interpreted false negative by IFA, the positive rates as detected by IB ranged from 5.9 to 7.7 %, respectively.**

*** * EUROLINE ANA profile 3 and/or EUROLINE systemic sclerosis profile (Euroimmun)**

Table 5.Cellular function(s) of target antigen proteins reacting with ANA

Protein	Function
SS-A/Ro-60 (60 kDa) [1]	Protein Ro-60 associates with small RNA molecules (mainly family Y, especially with the Y^5 RNA) to form ribonucleoprotein Ro ^{hY5}RNP.
SS-B/La (48 kDa) [2]	Associates with the small RNAs from Y family (from Y^1 to Y^4 RNPs, but not Y^5)
Ro-52 [3]	Associates with a heterogenic population of small nuclear (sn)RNAs rich of uridine (the U RNP family)
nRNP/Sm (U1 nRNP) [4]	Protein Sm* associate(s) with U1 RNA (U1 nRNP is formed)
PM-Scl-75 and/or PM-Scl-100 [5]	Mammalian analogues of 3´- 5´-exoribonucleases D and/or PH, which participate in the mRNA splicing
Scl-70 (95 kDa) [6]	Topoisomerase I (topo I)
CENP-B (centromere)** [7]	Centromere proteins participate in the anchoring of kinetochores to chromosomes
Ribosomal P-protein [8]	A complex of 3 proteins (P0, P1 a P2), which are components of the large ribosomal subunit (60S) and participate in protein synthesis [13] ;serological marker for neuropsychiatric SLE (NPSLE) [14]
Jo-1 [9]	Histidyl-tRNA synthase (80 kDa); amino-acetyl group linkage to tRNA
PCNA [10]	Subunit of DNA polymerase; interacts with cyclins (complex regulation of cell division)
Sp100 [11]	Protein(s) of so called nuclear bodies, which are ubiquitin antagonists (also called SUMO, small ubiquitin modifiers).
Sm***	Several polypeptides associating with the U1 RNA.
AMA-M2 [12]	Mitochondrial matrix protein, pyruvate dehydrogenase
Histones H2, H3 and H4	Basic proteins which associate with the dsDNA (by nucleosome formation)
Histon H1	Regulatory dsDNA binding protein (released from euchromatin)
Nucleosome	The basic chromatin subunit (dsDNA segment to which histones H2, H3 and H4 bind)
Fibrillarin	U3 RNP

***at least 3 protein subunits; ***seven proteins (according to a patient´s name, Smith antigen)**

**** additional antigenic variants such as CENP-A, C, D, E and F were described, type B is the most frequent**

[1]Keech et al.,1996; Gendron, 2001; [2]Touloupi et al., 2005; [3] Pruijn et al., 1997; Schulte-Pelkum et al., 2009 [4]Miglionni et al., 2005; Reuter and Luhrman 1986; Wayaku et al., 2007; [5]Oddis et al., 1992; Mahler et al., 2006; Mahler and Raijmakers, 2007; [6]Kuhn et al. 1989; Dellavance et al., 2009; [7]Masumoto et al., 1989; [8]Gerli and Caponi, 2005; [9]Nishikai and Reichlin, 1980; [10]Maga and Hubscher, 2003 [11] Muratori et al., 2002; [12]Bogdanos et al., 2008; [13] the indirect immunofluorescence in HEp-2 cells is not a reliable screening test for the prediction of ribosomal P-antibodies (Mahler et al., 2008). [14] Eber et al., 2006 (not confirmed by others).

Table 6.The basic markers of ANCA (anti-neutrophil cytoplasmic antibody) associated lesions.

IFA pattern	Ethanol fixation	Formalin fixation	Target antigen	Recommended test	Disease
cANCA	Cytoplasmic granules	Cytoplasmic granules	PR3	IFA: granulocyte mosaic*, Biochip PR3 IB: MPO/PR3 strip MPO/PR3/GBM strip cANCA ELISA: PR3 (P3-hn-hr)***	Wegener`s granulomatosis (at least 66 % probability) AIC (autoimmunne cholangitis) and/or PSC (primary sclerotizing cholangitis), Table 7
pANCA	Perinuclear granules within the lobular protrusions of segemented nuclei, nuclear membrane thick accentuated	Cytoplasmic granules (no essential difference as compared to cANCA)	MPO	IFA: granulocyte mosaic*, Biochip MPO IB: MPO/PR3 strip and/or MPO/PR3/GBM strip MPO ELISA	Microscopic polyangiitis (MPA), (probability of 58 %), Eosinophilic granulomatous polyangiitis (EGPO) Ulcerative colitis (compare Table 8)
xANCA	Perinuclear fluorescence	No fluorescence	Lysozyme** Elastase Cathepsin G Lactoferrin	IFA in Hep-2 cells ELISA ELISA IFA: Lactoferrin-specific granulocytes	Ulcerative colitis (compare Table 8)

***Several modifications of the given mosaic are available: granulocytes (EOH), granulocytes (HCOH), Hep-2 cells, MPO biochip, PR3 biochip, GBM biochip; cell nuclei, lactoferrin specific granulocytes**

****in Hep-2 cells**

*****specific recombinant polypeptide (inactivated by A176 mutation) mixed with a native antigen isolated from human neutrophils**

Table 7. The serologic markers of autoimmune liver diseases

Disease	Autoantibody	Antigen	Detection (recommended test)
AIH1	ASMA	Actin, vimentin, desmin	IFA (liver mosaic)[1], VSM47 (transfected) cells
	ANA (homogenous pattern)	Histones, nucleosomes	IFA (basic mosaic) and IB (see also Table 2)
	pANCA	MPO	IFA (ANA mosaic), BIOCHIPS or IB test (MPO/PR3)
	anti-ASPG-R	ASPG-R	ELISA
	SLA/LP[2]	tRNA associated protein	ELISA (soluble liver antigen); IB: SLA/LP profile
AIH 2	LKM1	Cytochrome p450/2CYP2D6	IFA (liver mosaic), IB (autoimmune liver profile),
	LKM2	Cytochrome p450/2C9	ELISA
	LKM3	UDP-glucuronyl-transferase (UGT)	ELISA
	anti-ASPG-R	ASPG-R	IB strip: LKM1/LC-1/SLA-LP
	anti-LC-1 (liver cytosol 1)	formimino-cyclodeaminase (FTCD)	IB (autoimmune liver profile), ELISA
	anti-LM (liver microsome)	Cytochrome P450/IA2	
PBC	AMA-M2, Sp100 (see also Table 5)	Mitochondrial antigens	IFA (liver mosaic), IB (liver profile), ELISA anti-M2-3E[3]
AIC	ABCA	Intrahepatic bile canaliculi	IFA (liver mosaic)
	pANCA	(epithelium cells), MPO	IFA (ANA mosaic) and microdots or IB test for MPO/PR3

[1] contains: HEp-2 cells, primate liver section, rat kidney and liver section, rat stomach section and actin expressing VSM47 (transfected) cells;[2] former AIH3 [3] Dähnrich et al., 2009;

Table 8. Serologic markers in intestinal autoimmune disorders

Disease	Autoantibody	Antigen	IFA	IB	ELISA
Celiac disease	AEmA, ARA AGA DGP	tTG[1] gliadin, (alpha)-gliadin peptide deamidated (alpha)-gliadin peptide	Liver/esophagus/intestine Sections BIOCHIP GAF-3X (nonapeptide)[2]	Not available	Anti-tTG (IgA, IgG) GAF-3X (IgA, IgG)[2] deamidated gliadin peptides
Ulcerative Colitis	pANCA GAB[3]	lactoferrin (DNA bound), histone H1	Granulocyte smear formalin Fixed Adenocarcinoma line HT29-18N2	MPO/PR3 Strip Not available	MPO ELISA
Crohn´s Disease	PAB[4] ASCA[5]	CUZD1/rPAg1 (zymogen granule membrane protein) GP2/rPAg2 (acinus cell proteoglycan) GPI[6] Bacterial glycans Pseudomonas fluorescence Antigens	HEK296-CUZD1 transfected cells HEK293-GP2 transfected cells	Not available	 ALCA[7], ACCA[8], AMCA[9], anti-L[12], anti-C13 [10]anti-ompC, [11]anti-I2
Pernicious anaemia Atrophic gastritis	APCA[14]	Intrinsic factor H^+/K^+ ATPase	BIOCHIP, intrinsic factor coated Monkey stomach section, Parietal cells positive	Not available	ELISA (intrinsic factor) ELISA (H+/K+ ATPase)

[1] tissue transglutaminase; [2] gliadin related [2] nonapeptide, a 3 times repeated oligopeptide construct (gliadin specific) [3] goblet cell antibody [4](exocrine) pancreas acinus antibody [5]anti- *Saccharomyces cerevisae* antibody [6]Glycosyl-phosphatidyl-inosine; [7]anti-laminaribinoside carbohydrate antibody; [8]anti-chitobioside carbohydrate antibody; [9] anti-mannobioside carbohydrate antibody; [10]outer membrane porin C, [11]*Pseudomonas fluorescens* protein I2 [12]laminin [13]chitin [14]anti-patietal cell antibody

Table 9. Antibodies in autoimmune striated muscle diseases.

Disease	Autoantibody	Antigen target	Main test for detection	Comment
Polymyositis	Anti-Jo-1[1]	Histidyl-tRNA synthase[1]	IB (myositis specific antigen profile)*; Hep-20-10 cells[2]; ENA profile ELISA	Tsui and Siminovits, 1991, ASS**
JAMD[4]	Anti-p140	nuclear transcription, RNA metabolism[3]		Myopathy specific antibodies (MSA)
	Anti-CADM-140	Intracytoplasmic MDA-5[5]	ELISA, IB	
	Anti-Mi-2	DNA binding helicase		
	Anti-SAE	post-translation transcription modifier	ELISA, IB	
	Anti-TIF1-gamma	Transcription intermediary factor, cell differentiation	ELISA, IB	
Necrotizing myositis	Anti-SRP	Signal peptide recognition particle (SRP)	IB: myositis profile 3; autoimmune myopathies (15 antigens)	Idiopathic polymyositis (IPM)
MCTD[6]	Anti-U1nRNP Anti-U3RNP	nRNP/Sm U3RNP/fibrillarin	IB: Euroline ANA profile, Euroline ENA profile, monospecific ELISA	Sharp et al., 1972

[1]several other amino-acyl-tRNA synthases: (their reaction products bind to CCR5 chemokines and to toll-like rectors (TLR4) *primate heart muscle/iliopsoas muscle mosaic **anti-synthase syndrome [2]increased number of mitoses induced in a synchronized line; [3] 140 kDa receptor for RNA viruses, (IFN- gamma induction); [4]juvenile (or adult) autoimmunity based muscle disease [5]melanoma associated gene 5 encoded protein [6]mixed connective tissue disease

Table 10. Survey of the autoimmune lesions in squamous epithelium

Disease	target antigen	IFA staining*	IB	ELISA	remark
pemphigus vulgaris (PV)	desmoglein 3 (Dsg3) Dsg3 and Dsg1***	Spinal (middle) layer of squamous epithelium Spinal and subcorneal layers (intercellular spaces)	No	yes**	Mainly mucosal involvement Mucocutaneus (combined) involvement
Pemphigus folliaceus (PF)	desmoglein 1 (Dsg1)	Subcorneal localization in squamous Epithelium	No	yes**	Mainly cutaneous involvement
Bullous pemphigoid (BP)	BP180 (180 kDa) BP230 (230 kDa)	Basal squamous cells (at *lamina lucida* region) show fine granular (dispersed) fluorescence adjacent to BM	No	Yes****	

* primate skin and oesephagus sections; ** either recombinant Dsg3 or Dsg1 coated to microtiterplates; ***recombinant HEK cells are available expressing one of the target proteins (Dsg3 or Dsg1); ****recombinant BP180 coated to microtiterplates

Table 11. Autoantibodies in neural disorders

Autoantibody	Target antigen	IFA staining pattern	Additional test systems	Clinical relevance	Note
Anti-Hu (ANNA1)*	Hu protein (38 kDa)	Nuclei of CNS and PNS Neurons	IB: neuronal antigen profile; Paraneoplastic syndromes profile	Encephalomyelitis Neuropathy	Neuroblastoma SCLC[1]
Anti-Yo (ANNA2)*	NOVA (55 and 80 kDa)	Nuclei of CNS nerons	IB: neuronal antigen profiles	Myoclonus Syndromes	SCLC, breast Carcinoma
Anti-Yo (PCA-1)**	CDR2/CDR62 (34/64 kDa)	Cytoplasm of Purkynje Cells	IB: neuronal antigen profile(s)	Encephalomyelitis Neuropathy	Ovarian, breast and uterine carcinoma
PCA-2	Purkynje cell protein (280 kDA)	Dendrites of Purkynje Cells		Lambert-Eaton Myasthenic syndrome	SCLC, limbic/brain stem encephalopathy
Anti-Tr	DNER (notch-like epidermal growth factor receptor)	Dot-like fluorescence in the molecular layer	IFA: DNER antigen expressing HEK cells	Cerebellar Degeneration	Hodgkin´s lymphoma
Anti-glia antibodies (AGNA); anti- Sox	Tumor antigen SOX[2] Acidic glial nuclear protein	Bergmann´s glia nuclei Positive	IB: neuronal antigen profile(s)	Cerebellar Degeneration	SCLC, Lambert-Eaton myasthenic syndrome
Anti-PNMA (Ma1)	Ma (receptor) protein 37 kDa	Nucleoli of the neurons (in hippocampus)		Brain stem encephalopathy	Breast carcinoma
Anti-PNMA(Ma2)	Ma (receptor) protein 40 kDa		IB: neuronal antigen profile(s)		Testicular tumor
anti-MAG	Myelin associated glycoprotein	Peripheral nerve (annular) fluorescence	Not available	Neuropathy	Paraproteinemia (IgM)
Anti-AQP- 4	Aquaporin-4 protein	Virchow-Robin space along with small arteries	Transfected HEK cells expressing AQP	Neuromyelitis optica, myelitis	Devic´s syndrome
Anti-amphiphysin	Amphiphysin 128 kDa	Presyptic nerve endings, cerebellar sections	IB strip with amphiphysin protein	Paraneoplastic Syndrome	Also stiff person sy SCLC, breast carcinoma
Anti-GAD	Glutamic acid decarboxylase	Patchy fluorescence in *stratum granulosum*	GAD IB strip	stiff person Syndrome	GAD ELISA
Anti-GABA B-receptor	GABA B-receptor protein	Granular fluorescence of *stratum moleculare*	GABA B-receptor expressing HEK cells	Limbic encephalopathy	SCLS, paraneoplastic syndrome
Anti-NMDA receptor	NMDA receptor protein	Neuropil staining, *stratum moleculare* and *stratum granulosum*	Transfected HEK cells (expressing NR-1)	Paraneoplastic Syndrome	Teratomas (ovarian, testicular)

[1] small cell lung carcinoma; * anti-neuronal cell antibodies; **Purkynje cell autoantibodies;

