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Immune Complex Deposits as a Characteristic Feature of Mercury-Induced SLE-Like Autoimmune Process in Inbred and Outbred Mice

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1. Introduction

Systemic autoimmune diseases, such as systemic lupus erythematosus (lupus, SLE), rheumatoid arthritis and systemic sclerosis (scleroderma), occur in up to 5-8% of the human population. Based on frequency of occurrence, they are the third most common diseases after cardiovascular pathologies and cancers. The overwhelming majority of cases of autoimmune diseases are in women (Fairweather & Rose, 2004).

Nowadays, great attention is paid to studying the impact of environment on the development of autoimmunity and autoimmune diseases. This is due to the increased influence of anthropogenic factors on the quality of human life. There is a good reason to suppose that repeated exposure of people to low doses of heavy metal compounds may promote the development of such diseases (Havarinasab et al., 2009). Humans can be exposed to these noxious substances from atmospheric pollution, food, cosmetics (Bagenstose et al., 1999; Bigazzi, 1998; Pelletier et al., 1994; Rowley & Monestier, 2005), dental amalgams (Eneström & Hultman, 1995; Guzzi et al., 2008; Pigatto & Guzzi, 2010), thimerosal-containing vaccines (Mutter & Yeter, 2008) and through the regular contacts with materials in manufacturing processes (da Costa et al., 2008).

Mercury is one of the most global environmental pollutants, with human exposure to organic, inorganic, and elemental species of mercury occurring in many diverse settings. Relatively few studies exist in the literature on the relationship of mercury exposure and biomarkers of autoimmunity or autoimmune diseases in humans. Cases of mercury-induced autoimmune kidney disease mediated by immune complex (IC) deposition have been noted

historically in highly exposed populations (Gardner et al., 2010). A case-control study of scleroderma patients found an association between urinary mercury level and severity of the disease (Arnett et al., 1996). It is notable that the great artists who used paints containing mercury, suffered from autoimmune pathologies. Thus, P. Rubens, P.-A. Renoir, Robert O. Duffy suffered from rheumatoid arthritis, while P. Klee had systemic scleroderma (Pedersen & Permin, 1988).

Anti-nuclear (ANA) and anti-nucleolar (ANoA) autoantibodies presence in serum are used in the clinical diagnosis of lupus and scleroderma (Ho & Reveille, 2003; Kurien & Scofield, 2006), and some similarities have been noted between ANA/ANoA profiles in mercury-induced autoimmunity (HgIA) models and in some patients with scleroderma (Takeuchi et al., 1995). The possible influence of mercury on the human population was confirmed by the results obtained from the experiments with people exposed by mercury on the gold mine sites in Amazonian Brazil (Silbergeld et al., 2005; Silva et al., 2004). Exposure to mercury in these populations is related to the use of mercury in riverine small-scale artisanal gold mining operations, in which miners are directly exposed to inorganic and elemental mercury, and downstream communities can be exposed by consumption of fish contaminated by methylmercury in impacted watersheds (Gardner et al., 2010). Exposure to either methyl or inorganic mercury in such populations is associated with elevated titers of detectable ANA and ANoA (Alves et al., 2006).

Furthermore, through the use of rodent models, awareness of the direct effects of mercury on the immune system has increased. It is well known that the chronic administration of subtoxic doses of $HgCl_2$ (mercuric chloride) induces a SLE-like autoimmune disease in genetically susceptible inbred mice with $H-2^s$, $H-2^q$, and $H-2^f$ haplotypes or their hybrids (Hansson & Abedi-Valugerdi, 2003; Hultman et al., 1999; Reuter et al., 1989; Roether et al., 2002; Rowley & Monestier, 2005; Takeuchi et al., 1995). This HgIA is characterized by T-cell-dependent polyclonal activation of B-lymphocytes (Abedi-Valugerdi, 2008; Johansson et al., 1998), increased level of serum immunoglobulins (IgG1 and IgE) (Abedi-Valugerdi et al., 2000, 2008), production of ANoA and by the formation of IC in different organs impaired their functions (Arefieva et al., 2010; Bagenstose et al., 1999; Eneström et al., 1984; Havarinasab et al., 2008; Hultman et al., 1989; Robinson et al., 1997). Female mice tend to be more susceptible to HgIA, consistent with sex differences observed in autoimmune diseases in humans (Fairweather et al., 2008). It is important to notice, that thimerosal (constituent part of some vaccines for human) is equipotent to inorganic mercury in eliciting a lupus-like immune response in susceptible animals, but mice treated with methylmercury do not develop renal or systemic IC deposition (Haggqvist et al., 2005; Havarinasab et al., 2005, 2007; Havarinasab & Hultman, 2005).

The interesting thing is that the nucleolar 34-kDa protein fibrillarin is the major autoantigen in such autoimmune disorders either in mice or in human. Fibrillarin is one of the most evolutionarily conserved proteins and is involved in the early stages of maturation of ribosomal RNA (rRNA). This protein can often be a target of autoantibodies in various autoimmune disorders (Ho & Reveille, 2003; Rhodes & Vyse, 2007; van

Eenennaam et al., 2002; Yang et al., 2003): systemic scleroderma (58% of cases), SLE (39% of cases), rheumatoid arthritis (60% of cases). The presence of autoantibodies to fibrillarin in patients' blood is a suitable diagnostic marker for the early stages of autoimmune diseases development (Tormey et al., 2001). In addition, a recent case-control study reported that severely affected scleroderma patients with anti-fibrillarin antibodies (AFA) were more likely to have higher levels of mercury in urine as compared either to less severely affected cases without AFA or controls suggesting an etiologic role for mercury in this autoimmune disease (Arnett et al., 2000).

It is worth noting that, along with mercury which can induce the production of AFA among the genetically predisposed animals, some heavy metals such as silver (Abedi-Valugerdi, 2008; Hansson & Abedi-Valugerdi, 2003; Suzuki et al., 2011) and gold (Havarinasab et al., 2007, 2009) have the same ability. In addition, cadmium (Leffel et al., 2003), platinum (Chen et al., 2002) and lead (Tabata et al., 2003) are also known to have a negative impact on humans and animals. They induce/exacerbate the autoimmune processes in human and murine models of autoimmune diseases. However, unlike mercury, they do not cause such strong lymphoproliferation, polyclonal activation of lymphocytes and deposition of IC in kidneys - the most important features of autoimmune diseases.

Taken all the foregoing into account, it becomes clear why HgIA in mice is used as a model of human systemic autoimmune disorders for testing of immunosuppressive agents and for investigating of the molecular mechanisms of heavy metal-induced autoimmunity.

Following the modern hypothesis of a strong genetical predisposition to autoimmune diseases, the majority of animal experiments are conducted on inbred and genetically modified mice prone to mercury-induced or spontaneous autoimmunity. However, results obtained on such susceptible homozygous mice cannot be fully extrapolated on genetically heterogenous human population. That is why it is more correct to use different genetically heterogenous (outbred) mouse stocks as laboratory model in analysis of mercury exposure and consequences of it on humans. But, there are only a few studies supporting the idea that outbred mice are also susceptible to mercury and produce ANoA. It is earlier affirmed that development of ANoA production is controlled strictly by the class II of H-2 genes, i.e. only certain mouse strains with specific H-2 genotypes (e.g. H-2s and H-2q) produce ANoA upon exposure to mercury. Therefore, it was expected that mice carrying the heterozygous H-2 genotype will be highly resistant to mercury-induced ANoA production. In controversial, it has been recently found that chronic treatment with mercury induces production of ANoA in a large number of outbred ICR, NMRI and Black Swiss mice (Abedi-Valugerdi, 2008). Regarding this matter Dr. Abedi-Valugerdi has spoken his hypothesis about the absence of the particular genetical susceptibility to HgIA. It means that unlike in inbred mouse strains, H-2 heterozygosity does not confer resistance to mercury-induced ANoA production. In other words, environmental factors can induce autoimmunity in the absence of specific susceptible genes in members of a genetically heterogenous population. Thus, it allows to use outbred mice as suitable model for research of HgIA.

As mentioned above, one of the most significant features of $HgCl_2$-induced autoimmune process along with appearance of AFA is deposition of immune complexes in kidneys. It is necessary to notice that formation of IC in human kidneys is one of the consequences of heavy metals exposure or autoimmune diseases (Ohsawa, 1997; Markowitz & D'Agati, 2009). However, the exact localization and composition of the IC in kidneys, mechanisms of its formation and possible cytotoxic effects still remain poorly understood. We have performed this work to elucidate these questions and in order to examine whether IC are present in other organs of inbred mice. Another goal of our study was to discover whether AFA production and IC deposition, which are typical for mercury-induced autoimmunity in H-2s and H-2q inbred mouse strains, could be reproduced in outbred mice.

2. Material and methods

2.1. Mice

All studies presented were carried out in female inbred SJL/J and outbred CFW mice, which were 8 weeks old at the beginning of each experiment. Animals were obtained from Animal Breeding Facility-Branch of Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry (AAALAC accredited). All animals obtained were axenic at the time of arrival. The health status for these mice was confirmed by monitoring in the AnLab laboratory (Czech Republic). The mice were housed at the Animal Facilities of the Shemyakin-Ovchinnikov Institute of Bioorganic Chemistry of the Russian Academy of Sciences (Moscow, Russian Federation) under specific pathogen-free (SPF) conditions with access to tap water and standard chow *ad libitum*. All animal experiments described here were conducted in accordance with the "Good Laboratory Practice in Russian Federation" (Decree of the Minister of Health of RF #267, dated June 19, 2003), Section 11 of the Declaration of Helsinki of the World Medical Association (1964) and the International Guiding Principles for Biomedical Research Involving Animals (1985).

2.2. $HgCl_2$ treatment

A solution of 0.4 mg/mL $HgCl_2$ (Sigma-Aldrich, St. Louis, MO, USA) was prepared in sterile 0.9% NaCl solution (OJSC Biochemist, Saransk, Russia). Groups of mice were injected subcutaneously (s.c.) with either 0.1 mL of $HgCl_2$ (1.6 mg/kg body weight) or 0.1 mL of sterile 0.9% NaCl every third day for 6 weeks.

2.3. Blood and tissue sampling

Before the experiment and after 6 weeks of $HgCl_2$ treatment, blood was obtained by retro-orbital puncture under ether anesthesia. The collected blood was allowed to clot for 30 min at 37ºC, centrifuged (800g, 10 min) and the serum obtained was used or stored at -70ºC.

Then, the mice were euthanized in a CO_2 chamber. Their kidneys, liver and spleen were dissected out aseptically during the autopsy. The organ pieces were used for cryotomy or stored in liquid nitrogen until used.

2.4. Detection of anti-nucleolar antibodies

The presence of serum ANoA was determined by indirect immunofluorescence (IIF) using murine NIH/3T3 cells as a substrate. The cells were grown on glass coverslips in DMEM supplemented with 10% FCS, glutamine, penicillin, and streptomycin (Paneco, Moscow, Russia) at 37ºC in a 5% CO_2 / 95% air atmosphere. Coverslips with attached cells were washed in PBS and fixed with 4% paraformaldehyde (PFA) in PBS for 15 min at room temperature, rinsed in PBS three times for 10 min, and permeabilized with 0.1% Triton X-100 in PBS for 10 min on ice, then washed in PBS four times for 5 min. The cells were incubated with the primary antibodies (murine sera from autoimmune and control mice) diluted 1:100 to 1:10000 in PBS and kept in the dark in a moist chamber for 45 min. Then, the cells were washed in PBS three times for 10 min, and incubated with Cy2-conjugated goat anti-mouse immunoglobulin G (IgG) antibodies (Jackson ImmunoResearch Lab., West Grove, PA, USA), diluted 1:200, for 45 min under the same conditions. Control cells were processed at the same time and in the same way, except that PBS was used instead of the murine sera. No stained structures were seen in the controls. Then, the cells were washed again three times for 10 min, incubated in 1 μg/mL DAPI (4',6'-diamidino-2'-phenyindole) (Sigma-Aldrich, St. Louis, MO, USA) solution at room temperature for 10 min and then mounted on slides with Mowiol containing DABCO (1,4-diazabicyclo[2.2.2]octane) (Sigma-Aldrich, St. Louis, MO, USA). During the experimental period, slides were stored at 4ºC in the dark. The titer of ANoA was defined as the highest serum dilution that gave specific nucleolar staining (Havarinasab et al., 2008).

The cells were examined using an Axiovert 200 epifluorescence microscope (Carl Zeiss, Oberkochen, Germany) with PlanNeoFluar 40×/NA 0.75, Fluar 100×/NA 1.25, and AchroPlan 100×/NA 1.3 objectives. Images were obtained using a 13-bit monochrome camera (CoolSnap_cf; Roper Scientific, Tucson, AZ, USA).

2.5. Analysis of immune complexes in kidneys

The presence of renal IC in kidneys of inbred and outbred mice was detected by direct immunofluorescence (DIF). The slides with attached 5-μm thick cryosections (Microm HM 525, ThermoFisher Scientific, Waltham, MA, USA) were washed in cold PBS and then air-dried or fixed under various conditions: in absolute acetone/methanol for 5 min or in 4% PFA in PBS for 15 min. Some sections were then air-dried after fixation in absolute acetone or methanol. After incubation in 4% PFA, slides were washed three times for 10 min and permeabilized with 0.1% Triton X-100 in PBS for 10 min on ice, then washed in PBS four times for 5 min. After that, the sections were incubated with serial dilutions of either a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse total Ig (IgG + IgA + IgM) antibodies (Imtek, Moscow, Russia) and/or rabbit anti-mouse complement factor C3 primary antibodies (Abcam, Cambridge, UK), diluted 1:10, and Texas Red-conjugated donkey anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Lab, West Grove, PA, USA), diluted 1:400, in the dark in the moist chamber for 45 min. The initial dilution for FITC-conjugated antibodies was 1:50. The end-point titer for total immunoglobulins (Ig) was

defined as the highest dilution that gave specific staining. Then, the slides were washed three times for 10 min, incubated in DAPI solution, as described above, and then mounted with Mowiol containing DABCO. During the experimental period, the slides were stored at 4ºC in the dark.

To visualize renal blood vessels varying in size and colocalize them with IC we used additional staining of organs with special dye Col-F (kindly furnished by Dr. Jerzy Dobrucki), which reveals collagen and elastin fibers that are part of the coats and elastic membranes of blood vessels. Halves of organs were washed in DMEM and put in Col-F dye solution in DMEM at 37ºC for 1 h. Then, the pieces of kidney were rapidly washed again, and cut for cryosections as described previously. To remove damaged/non-specifically stained tissue, the first 50-µm thick section was discarded. Fixed in 4% PFA cryosections were then washed and incubated according to the DIF method described above.

With the aim of distinguishing between proximal and distal renal tubules and colocalizing them with IC, we performed a staining assay using phalloidin-tetramethylrhodamine B isothiocyanate (TRITC; Sigma-Aldrich, St. Louis, MO, USA). This is a fluorescent phallotoxin that can be used to detect actin-rich structures, such as the brush border of the proximal renal tubules. After fixation cryosections were incubated with a mix of 5 µg/mL TRITC-conjugated phalloidin solution and FITC-conjugated goat anti-mouse total Ig antibody in the dark in a moist chamber for 45 min, washed, incubated in DAPI and mounted with Mowiol.

The slides were examined using Axiovert 200 epifluorescence and confocal LSM510 microscopes (Carl Zeiss, Oberkochen, Germany). Images were obtained using a 13-bit monochrome camera (CoolSnap$_{cf}$, Roper Scientific, Tucson, AZ, USA).

2.5.1. Detection of immunoglobulin isotypes as components of renal IC

Presence of IgG1, IgG2a and IgM immunoglobulins in kidneys of mercury-treated and control mice were determined using DIF, as described previously. To distinguish between IgG and IgM classes, we used fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse total Ig (IgG + IgA + IgM) antibodies (Imtek, Moscow, Russia) in combination with TRITC-conjugated goat anti-mouse IgM secondary antibodies (Jackson ImmunoResearch Lab, West Grove, PA, USA).

To evaluate the presence of IgG1 and IgG2a immunoglobulin isotypes, we used TRITC-conjugated goat anti-mouse IgG antibodies (Jackson ImmunoResearch Lab, West Grove, PA, USA) in combination with FITC-conjugated goat anti-mouse IgG1 or IgG2a secondary antibodies (SouthernBiotech, Birmingham, AL, USA). The dilution for all the antibodies mentioned was 1:50.

2.5.2. Glomerular cell death assessment

The terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling (TUNEL) method was performed on the kidney cryosections to visualize apoptotic glomerular cells to assess

the cytotoxicity of immune deposits. It is widely known that the most common biochemical property of apoptosis is the endonucleolytic cleavage of chromatin. This method identifies apoptotic cells *in situ* using terminal deoxynucleotidyl transferase (TdT) to transfer biotin-dUTP to the free 3'-OH of cleaved DNA. The biotin-labeled cleavage sites were then visualized by reaction with fluorescein-conjugated avidin (avidin-FITC). TUNEL staining was conducted with a kit (TUNEL Apoptosis Detection Kit, Upstate Biotechnology, Lake Placid, NY, USA) according to the manufacturer's protocol. Next, the slides were incubated in DAPI solution, then mounted with Mowiol containing DABCO. The cells were then observed using a fluorescence microscope. Sections treated with 1 μg/mL bovine DNase I were used as a positive control for the TUNEL assay and DNA fragmentation.

2.6. Analysis of immune complexes in liver and spleen

The presence of IC in liver and spleen of inbred and outbred mice was also detected by DIF. The slides with attached cryosections were washed in cold PBS and then fixed in 4% PFA in PBS for 15 min. After that, the slides were prepared as described above.

For the blood vessels detection we used the same technique as it was described for kidneys.

2.7. Assay for *in vivo* binding of mercury-induced ANoA to the nucleoli of living cells

The colocalization of the mercury-induced ANoA with nucleolar protein fibrillarin within the cell nucleoli in mercury-treated outbred mice was detected by the IIF method. Briefly, the sections were fixed in 4% PFA, washed, permeabilized and incubated with 1:50 dilution of the primary rabbit polyclonal anti-fibrillarin antibodies (Abcam, Cambridge, UK) in the dark in the moist chamber for 45 min and washed again. FITC-conjugated goat anti-mouse total Ig (IgG + IgA + IgM) antibodies (Imtek, Moscow, Russia) in combination with Texas Red-conjugated donkey anti-rabbit IgG secondary antibodies (Jackson ImmunoResearch Lab, West Grove, PA, USA), diluted 1:100 and 1:200 respectively, were used as secondary antibodies. Then the slides were washed again, stained with DAPI and mounted with Mowiol according the IIF technique.

2.8. Statistical analysis

Serum levels of ANoA and IC titers are all expressed as means±standard deviation. Differences between these parameters for the control and Hg-treated groups were analyzed for statistical significance using the Mann-Whitney U-test. P values < 0.05 were considered to indicate statistical significance.

3. Results

3.1. HgCl₂ induces ANoA production in inbred and outbred mice

To test the ability of mercury to induce ANoA production in inbred and outbred mice, sera obtained from experimental and control mice were tested using an IIF technique. Analysis of

sera obtained from mice at the end of the experiment (after 6 weeks of HgCl₂ treatment) showed that mercury was able to induce ANoA, in contrast to the saline-injected controls. The mean titer of ANoA in group of mercury-treated inbred mice was about 1520, in group of outbred mice - 10545 (Table 1). The pre-bleed sera from all groups of animals were ANoA-negative.

Mouse stock	Treatment	No. of mice	Titer of ANoA	Titer of IC		
				Glomerular mesangium	Blood vessel walls	Proximal tubules
Inbred **SJL/J**	6 weeks of **HgCl₂**	5	1520±1772[1,2]	17600±7838[2]	14400±3200[2]	4000[2]
	6 weeks of NaCl	4	0	1400±400	0	1400±400
Outbred **CFW**	6 weeks of **HgCl₂**	12	10545±3236[2]	6083±2018[2]	4750±1868[2]	13167±8462[2]
	6 weeks of NaCl	12	0	1700±249	0	2000±1083

[1] The numbers refer to mean reciprocal titer ± standard deviation; [2] $P < 0.05$, vs. saline-treated controls (Mann-Whitney U-test).

Table 1. Titers of serum ANoA and IC in kidneys of autoimmune and control mice.

Sera were incubated on NIH/3T3 cells followed by incubation with FITC-conjugated anti-mouse immunoglobulin antibodies; they characteristically stained nucleoli of interphase cells and peripheral chromosomal material (PCM) in mitotic cells (Fig. 1). Such stained regions matched the areas in which the major autoantigen in HgIA, fibrillarin, was revealed.
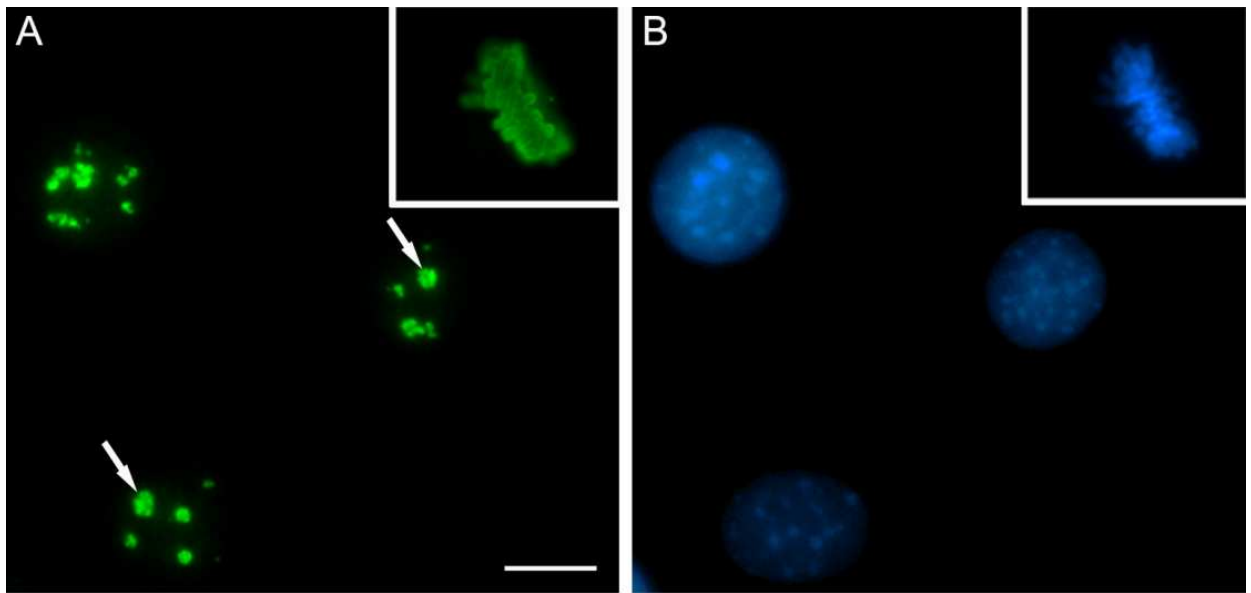

Figure 1. The nucleolar protein fibrillarin in murine NIH/3T3 cells is identified by serum from SJL/J autoimmune mouse treated with HgCl₂. A - indirect immunocytochemistry reveals fibrillarin in the nucleoli of interphase cells (*arrows*) and in the peripheral chromosomal material of metaphase cells (*insert*); sera from CFW autoimmune mice reveal the same staining patterns. B - DAPI staining of interphase nuclei and metaphase chromosomes. Scale bar - 10 μm.

So, we have concluded that six weeks of HgCl$_2$ treatment to female SJL/J and CFW mice resulted in strong ANoA production.

3.2. HgCl$_2$ induces heavy immune complex deposition in kidneys of inbred and outbred mice

3.2.1. Identification of glomerular IC in murine kidneys

Glomerular IC were revealed with all variants of tissue treatment (Table 2). However, on fixed cryosections, the general tissue morphology was better than on unfixed sections. In all cases, immune deposits were seen in the form of granules, which in places with the highest congestion merged and looked like brightly fluorescing spots (Fig. 2). Under epifluorescence and confocal microscopes, immune deposits often repeated the form of the mesangial cells and were clearly visible in the plane of the nuclei, as revealed using the dye DAPI or phase contrast (Fig. 2, 3). Additionally, only fixing with 4% paraformaldehyde strongly reduced the background fluorescence and increased clarity when looking at a tissue; the borders of the immune deposits appeared to be much sharper than with the other fixing techniques. Additionally, fixed sections could be stored for about 3 months at -70ºC without any appreciable loss of staining ability.

Localization	Type of fixation					
	Air-drying	Acetone	Acetone/ air-drying	Methanol	Methanol/ air-drying	4% PFA
Glomerular mesangium	+[1]	+	+	+	+	+
Blood vessel walls	+	+	+	+	+	+
Proximal tubules	-[2]	-	-	-	-	+

[1] Presence of IC; [2] absence of IC.

Table 2. IC in kidneys of HgCl$_2$-treated mice, revealed with various fixing conditions.

Our results showed a significantly increased titer of immunoglobulins in the glomerular mesangium in kidneys of HgCl$_2$-treated animals compared with control mice (Table 1). The HgCl$_2$-treated groups of mice showed a mean titer of mesangial Ig of about 17600 (inbred mice) and 6083 (outbred mice). The saline-injected control groups showed only 1400 (inbred) and 1700 (outbred) mean titers of secondary antibodies.

Moreover, we also have found the deposition of C3 component of complement system as part of glomerular IC (Fig. 4). It is necessary to notice that such C3 deposits do not always colocalize with regions containing immunoglobulins. This means that there are such areas in glomeruli where only C3 is revealed, but at the same time immunoglobulins are not seen. The saline-injected control mice were completely devoid of C3 deposits.
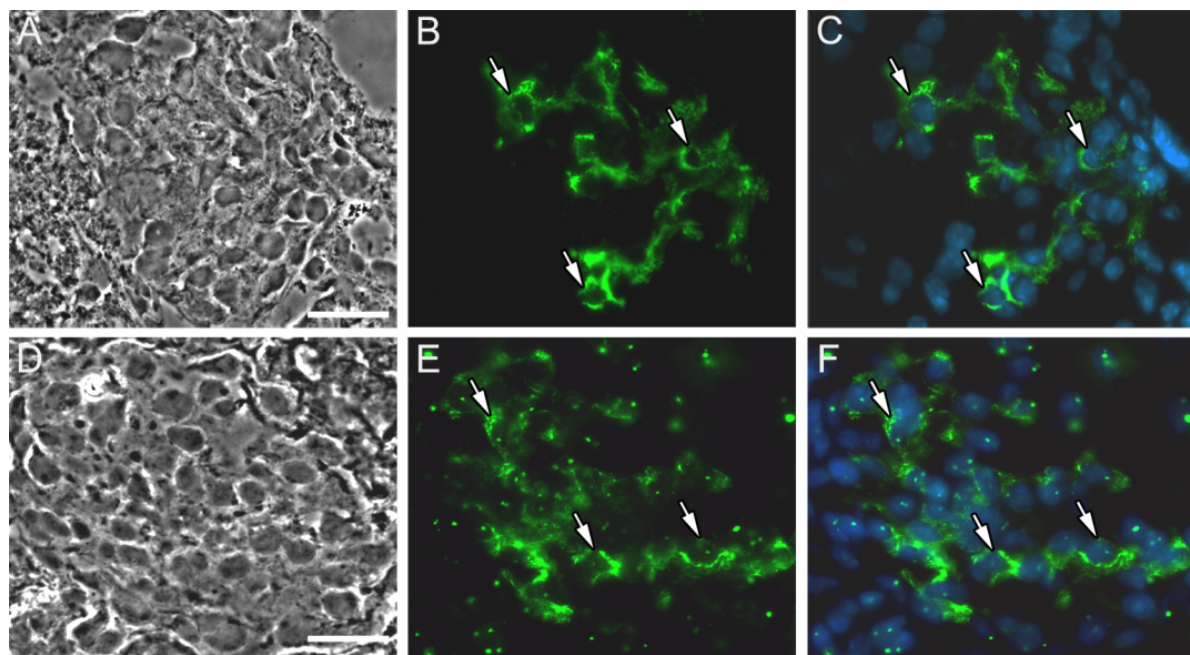
Figure 2. Immunohistochemical detection of immunoglobulins in glomeruli of autoimmune inbred (A-C) and outbred (D-F) mice. *Left column* - phase contrast, *middle column* - total immunoglobulin staining, *right column* - merge of immunoglobulin staining and nuclear chromatin DAPI–staining. *Arrows* point to glomerular cells in which it can be clearly seen that immunoglobulins are located in the cytoplasm around the cell nucleus. Immunoglobulins in the nuclei of outbred mice are also clearly seen. Scale bar - 30 μm.
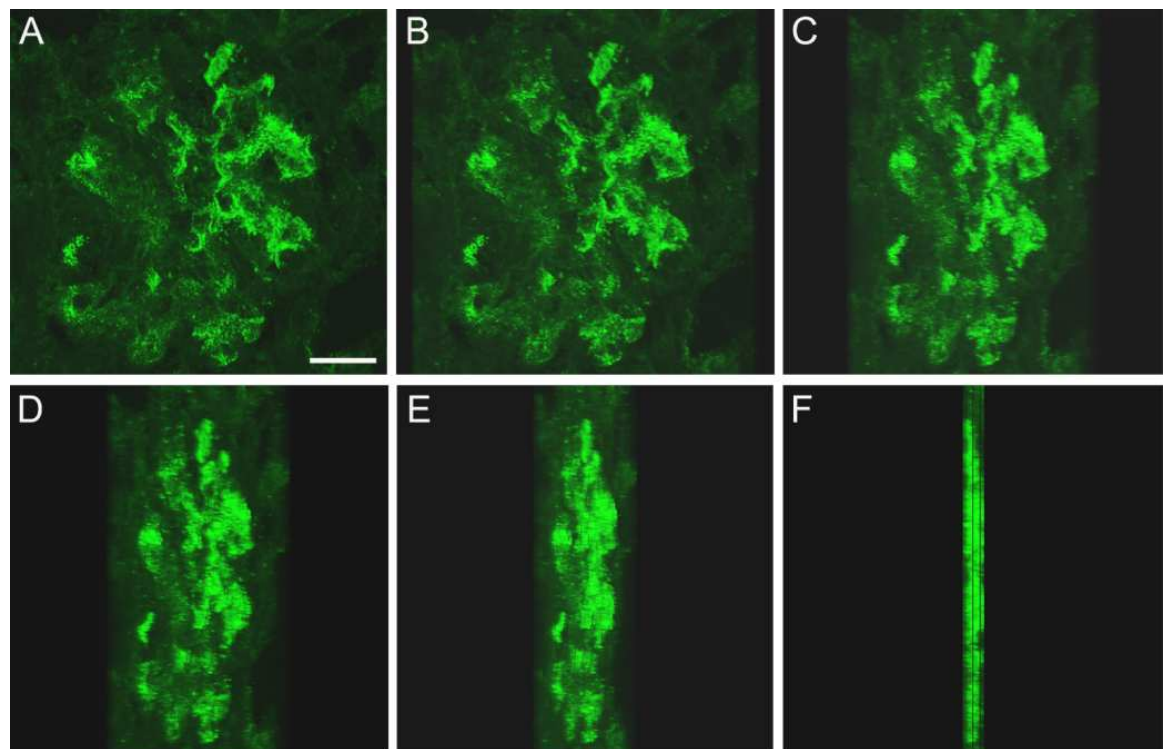

Figure 3. Visualization of immunoglobulins in glomerulus of autoimmune mouse under confocal laser scanning microscope. A–E: glomerular area reconstructed on the base of serial optical sections (counter-clockwise rotation model); F: side-view of the reconstructed area (thickness of this area is about 7 μm). Scale bar - 30 μm.
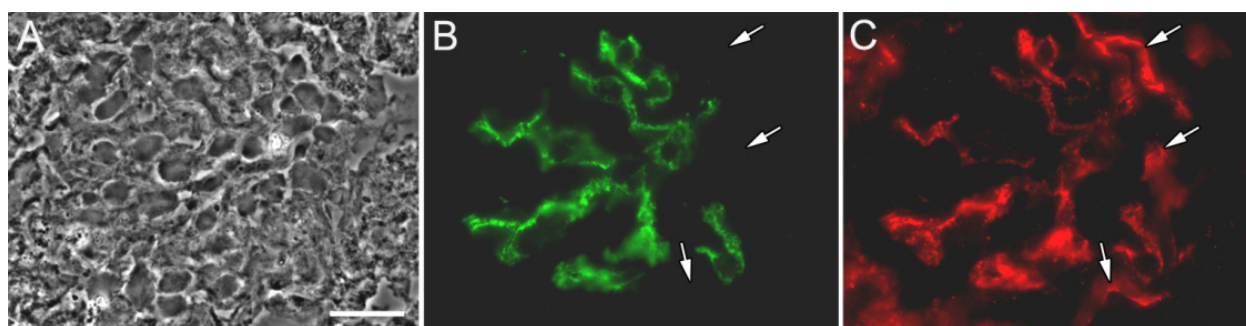
Figure 4. Localization of immunoglobulins and the C3 component of the complement system as components of immune deposits in glomerulus of autoimmune mouse after HgCl₂ treatment. A - phase contrast, B - total immunoglobulin staining, C - staining of C3 component of the complement system. *Arrows* show the C3-containing areas without immunoglobulins. Scale bar - 30 μm.

3.2.2. Identification of vascular IC in murine kidneys

Additionally, only the mercury-treated mice, not the control groups (Table 1), showed IC (Ig + C3) in the walls of renal blood vessels with all variants of tissue treatment (Table 2). This finding was confirmed by colocalization of IC with collagen and elastin fibers that are part of the coats and elastic membranes of blood vessels, which we revealed using the Col-F dye (Fig. 5). Comparison of places with immune deposit localization with collagen and elastin staining allowed us to conclude that IC were present in both the endothelial zone of vessels and in different layers of the basement of the vessel walls. Using the Col-F dye allowed us to conclude that deposits were present in all renal vessel walls, regardless of their size. Moreover, Col-F revealed even vessels that were not seen clearly with phase contrast. Similarly, glomerular IC could be seen in the form of granules, which in places with the highest concentration merged and looked like brightly fluorescing spots.

The mean titer of vascular Ig in the kidneys of the HgCl₂-treated groups of mice reached 14400 (inbred mice) and 4750 (outbred mice). The saline-injected control groups were completely devoid of deposits (Table 1).

3.2.3. Identification of tubular IC in murine kidneys

Immune deposits in renal tubules were seen out only when using 4% PFA as a fixative (Table 2). IC were seen in discrete granules of approximately equal size (about 1 μm) located in tubular epithelial cells (Fig. 6B, C).

We note that part of the renal tubules contained immune deposits whereas another part had none. To determine in which type(s) of renal tubules IC were present, we performed a combination of IHC analysis and staining with phalloidin-TRITC. As is well-known, phalloidin binds to actin, the basic structural component of the brush border, which is present in proximal renal tubules and is not expressed in distal parts. These results suggest that immune deposits were seen only in the proximal renal tubules, with a brush border, and not in the distal tubules, without a brush border (Fig. 6A, B).

Interestingly, the tubular IC consisted of immunoglobulins, but not the C3 component of complement. The mean titer of such deposits in proximal tubules was nearby 4000 (inbred) and 13167 (outbred). Besides, elevated titer of IC in the proximal tubules in outbred mice (compared with inbred mice) correlated with elevated titer of ANoA in their blood. Furthermore, the control group exhibited a lower mean titer of IC - 1400 for inbred mice and 2000 for outbred mice (Table 1). To our knowledge, this is the first report of immune deposits in the proximal renal tubules.
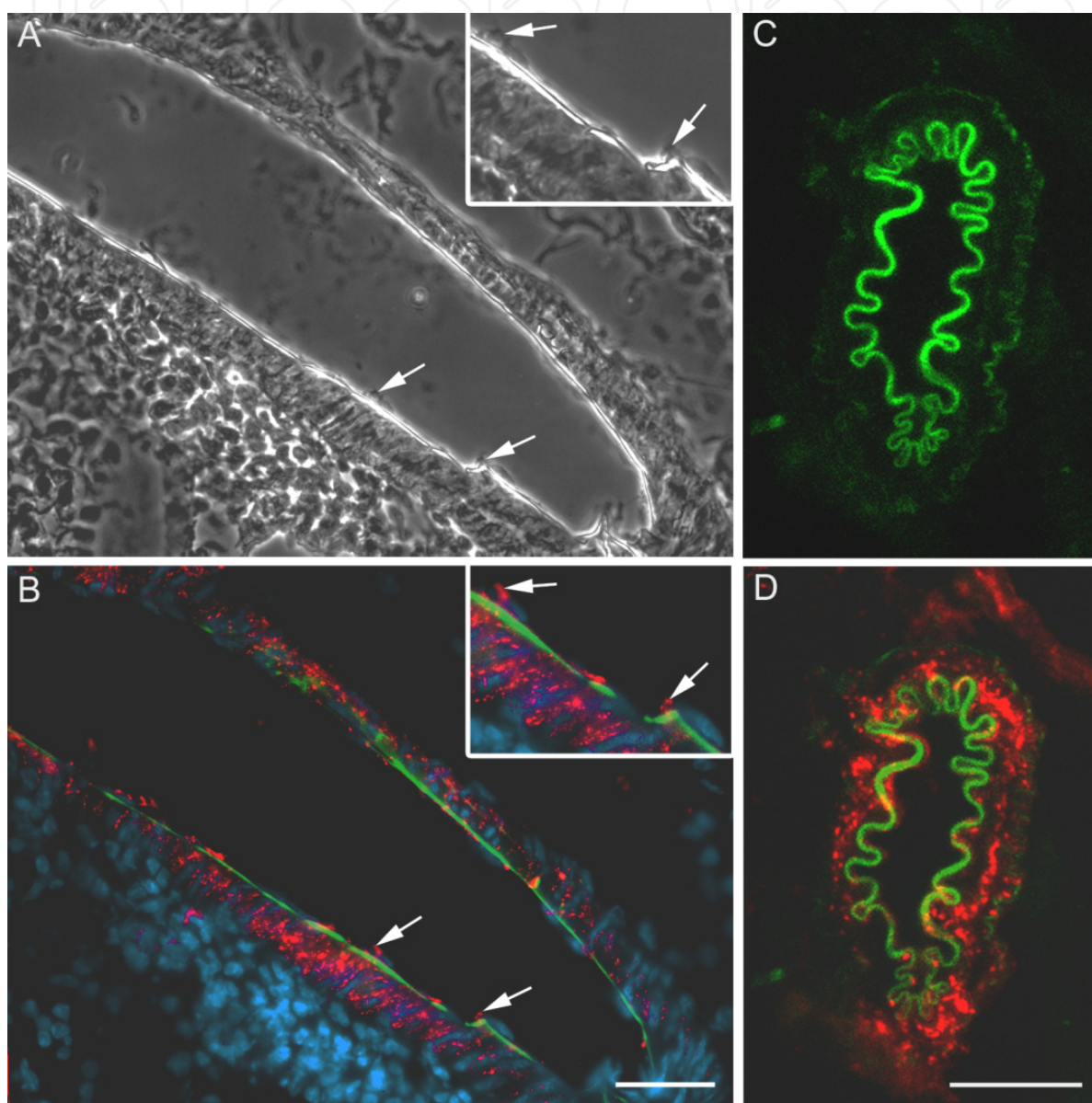

Figure 5. Immunoglobulins in renal blood vessels of varying sizes in autoimmune mice revealed with Col-F - a dye specific for collagen and elastic fibers. A, B - immunoglobulins in the blood vessel, which is clearly seen under phase contrast: A - phase contrast, B - staining with antiimmunoglobulin antibodies (*red*), Col-F dye (*green*) and nuclear chromatin staining with DAPI (*blue*); *inserts, arrows* - immunoglobulins in the endothelial part of this vessel. C, D - immunoglobulins in the blood vessel, revealed only by the Col-F dye: C - Col-F dye, D - staining with anti-immunoglobulin antibodies (*red*) and Col-F dye (*green*). Scale bar - 50 μm.
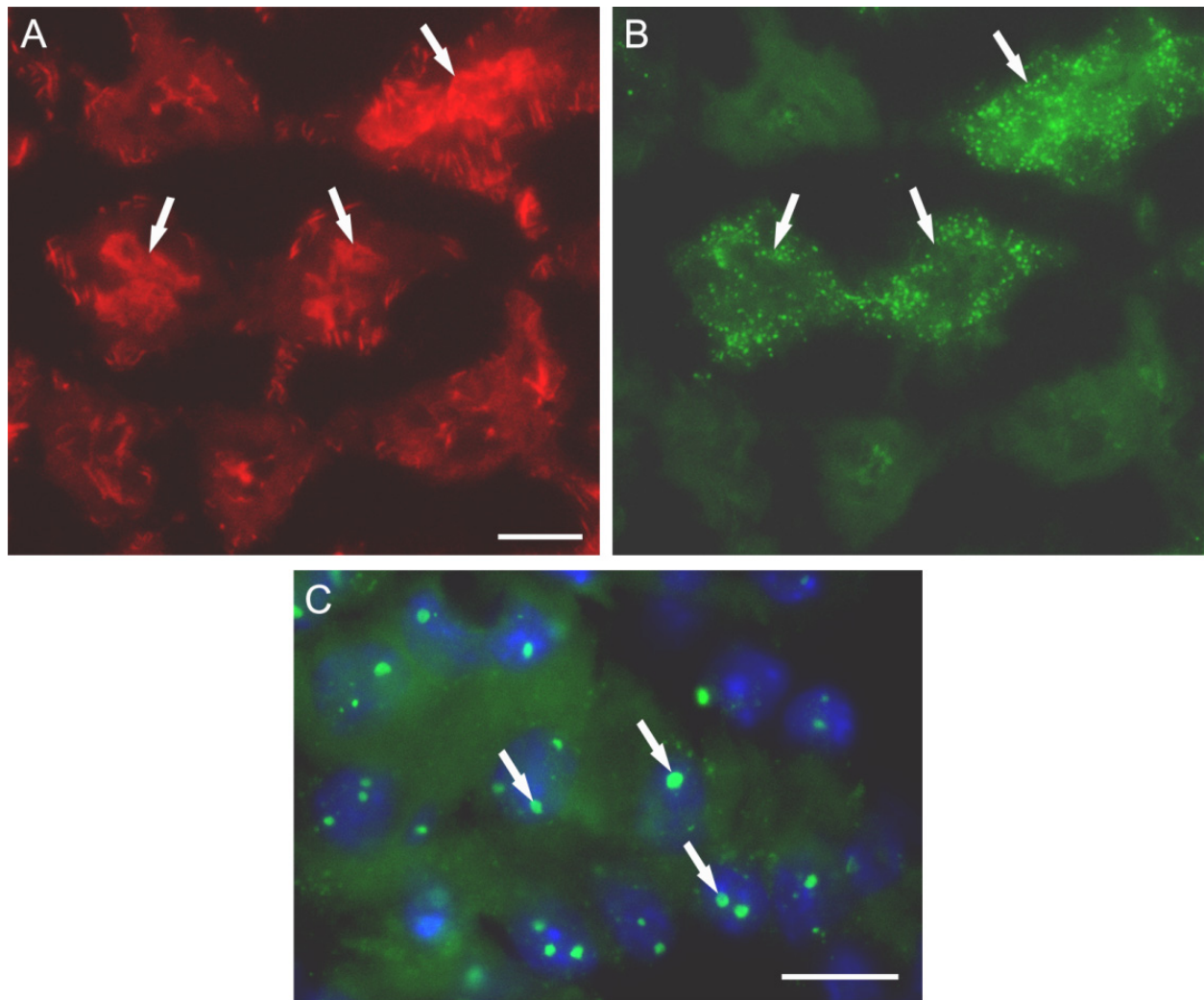
Figure 6. Localization of immunoglobulins in proximal renal tubules in inbred (A, B) and outbred (C) mice. A - revealing of brush border (*arrows*) in proximal tubules of inbred mice with the help of Phalloidin-TRITC staining, B - revealing of immunoglobulins in cytoplasm of epithelial cells in proximal tubules (*arrows*) of inbred mice; scale bar - 30 µм. C - revealing of immunoglobulins in cytoplasm and nuclei (*arrows*) of epithelial cells in outbred mice; scale bar - 20 µm.

3.2.4. Identification of immunoglobulin isotypes as components of IC in kidneys of inbred and outbred mice after HgCl₂ treatment

To understand which classes and isotypes of immunoglobulins are found in IC in different parts of murine kidneys, we performed combined multicolor DIF. Our results showed that immunoglobulin class G (IgG) occurred in all locations of IC: in glomeruli, blood vessel walls, and proximal tubules of autoimmune mice, and in glomeruli and proximal tubules of control mice. Immunoglobulin class M (IgM) was seen in glomeruli, proximal tubules, vessel walls of outbred autoimmune mice, and only in the glomeruli of both inbred autoimmune and control mice (Table 3). We did not assess whether immunoglobulin class A (IgA) was part of the immune complexes.

Mouse stock	Localization	IgG + IgM + IgA		IgG1		IgG2a		IgM	
		control	HgCl₂	control	HgCl₂	control	HgCl₂	control	HgCl₂
Inbred SJL/J	Glomerular mesangium	+[1]	+	+	+	-[2]	±[3]	+	+
	Blood vessel walls	-	+	-	+	-	-	-	-
	Proximal tubules	+	+	+	+	-	+	-	-
	Nucleoli	-	-	-	-	-	-	-	-
Outbred CFW	Glomerular mesangium	±	+	±	+	-	-	±	+
	Blood vessel walls	-	+	-	+	-	-	-	±
	Proximal tubules	+	+	±	+	-	±	-	+
	Nucleoli	-	+	-	+	-	+	-	-

[1] Presence of IC; [2] absence of IC; [3] slight deposits.

Table 3. Occurrence of different immunoglobulin isotypes as components of IC in kidneys of autoimmune and control mice.

Next, we tried to determine the IgG isotypes in IC. The results showed that the mesangial IgG deposits were dominated by the IgG1 isotype, but also contained IgG2a, consistent with Havarinasab et al. (2008). At the same time, we found only the IgG1 isotype in renal vessel wall deposits and both IgG1 and IgG2a isotypes in proximal tubule deposits (Table 3). The saline-injected control mice showed an absence of IgG2a deposits.

3.2.5. Assessment of possible IC toxicity in murine kidneys

To analyze the possible cytotoxicity of renal IC, we used the TUNEL method, which reveals fragmentation of DNA, a sign of cell destruction, *in situ*. The results showed that in glomeruli of both experimental and control animal groups, there was no significant increase in TUNEL-positive (i.e., apoptotic) cells.

3.3. Identification of IC in liver and spleen of inbred and outbred mice after HgCl₂ treatment

In the liver, a granular fixation of the anti-Ig antibodies was observed in the blood vessel walls of mercury-treated mice and, in contrast with earlier studies, in the liver hepatocytes in all groups of mice (Fig. 7). As in the kidneys, our results showed a significantly increased titer of Ig in the hepatocytes of HgCl₂-treated animals compared with control mice (not shown).

In the spleen, an intense granular pattern of the anti-Ig antibodies fixation was observed in the blood vessel walls and in the cells of lymphoid follicles, germinal centers, marginal zones and periarterial lymphatic sheaths (PALS) in mercury-treated mice (Fig. 8A, B).

White pulp of control animals had very rare and small germinal centers (these centers are known to appear during the Th2-dependent immune reactions). In contrast, germinal centers after the mercury chloride treatment were enlarged, prominent and quite frequent in all mice. Therefore, clear morphological attributes of Th2-antibody-producing immune response had been induced by the mercury chloride treatment in spleen.
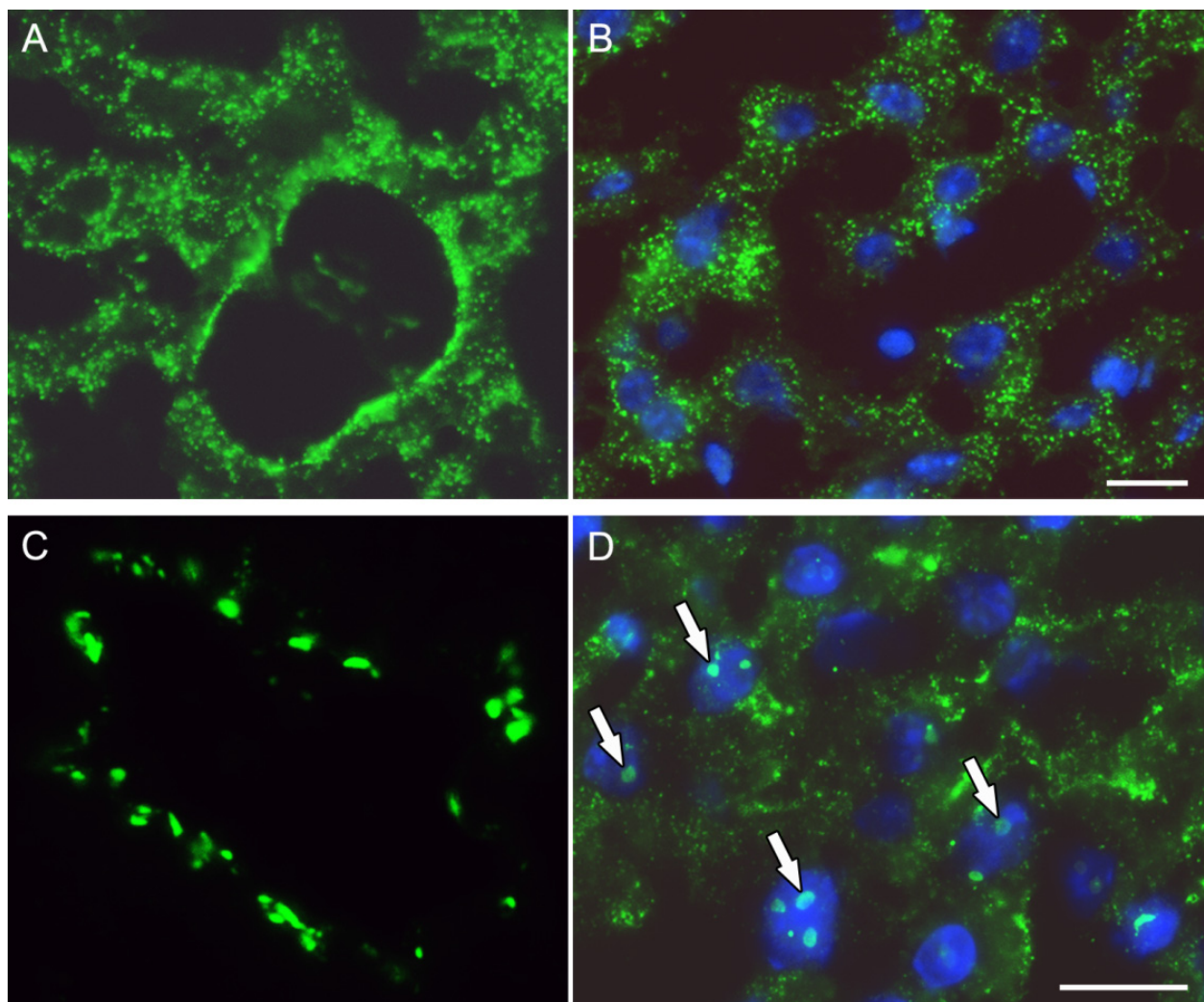

Figure 7. Immunohistochemical detection of immunoglobulins in liver of autoimmune inbred (A-B) and outbred (C-D) mice. A, C - immunoglobulins in liver blood vessels (total immunoglobulin staining); B, D - immunoglobulins in hepatocytes (merge of immunoglobulin staining and nuclear chromatin DAPI–staining), *arrows* point to nuclei in which immunoglobulins can be clearly seen. Scale bar - 20 μm.
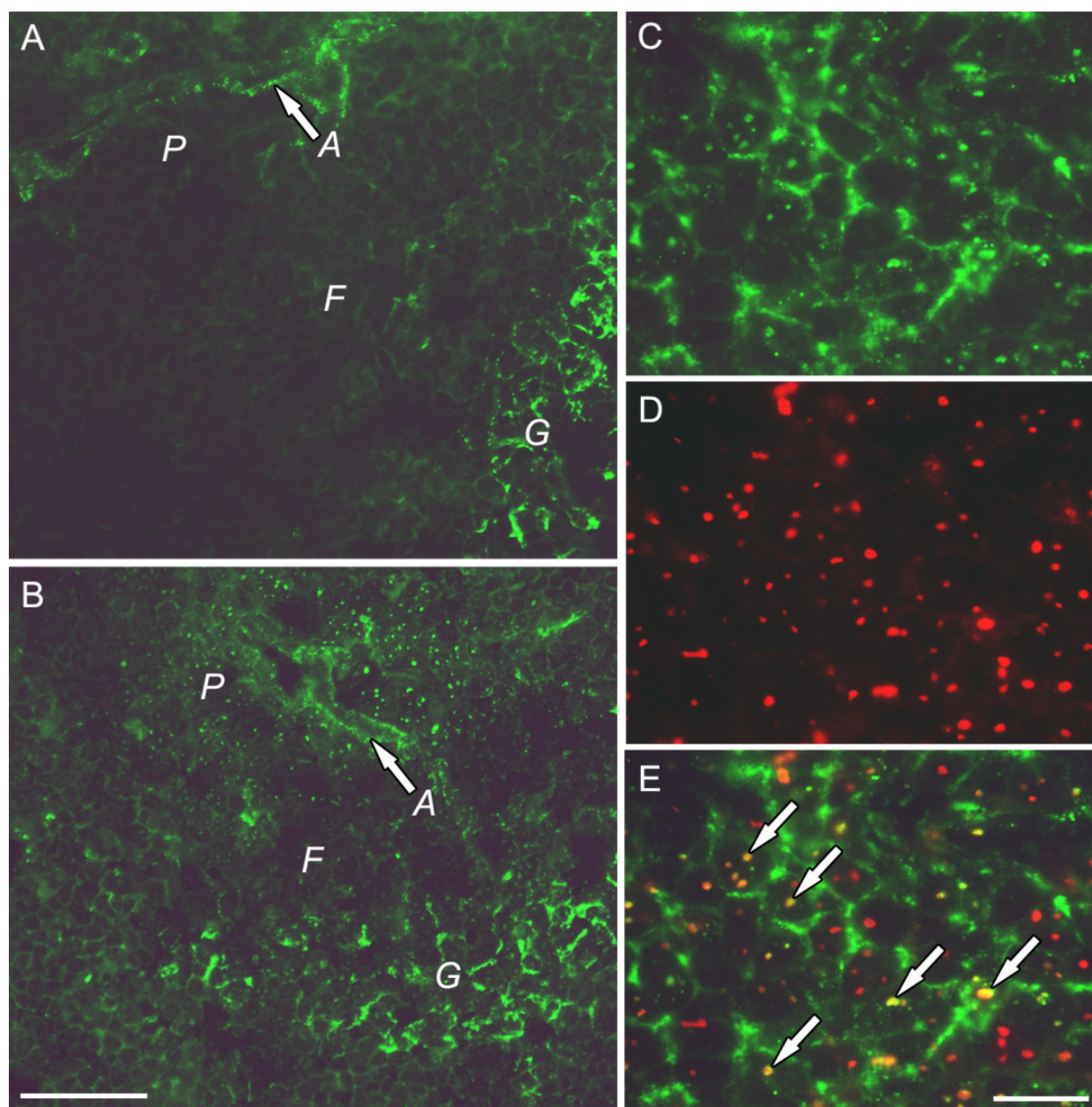
Figure 8. Immunohistochemical detection of immunoglobulins in spleen of autoimmune inbred (A) and outbred (B-E) mice. A, B - immunoglobulins in spleen blood vessels (*A-central artery, arrows*) and white pulp (*F-follicle, G-germinal center, P-PALS*), scale bar - 50 μm. C-E - results of colocalizing procedure: C - total immunoglobulin staining, D - staining with antifibrillarin antibodies, E - merge of immunoglobulin and fibrillarin staining patterns (yellow - *arrows*), scale bar - 20 μm.

3.4. Mercury-induced ANoA bind to the nucleoli of the kidney, liver and spleen cells in outbred mice *in vivo*

After analyzing the organs such as kidney, liver and spleen in all animals after 6 weeks of $HgCl_2$ treatment, we noticed some differences in the staining patterns in tissues from inbred and outbred mice following the method of DIF. As shown in Fig. 2, 6, 7, 8 most of the cells in

the kidney, liver and spleen sections of mercury-treated outbred mice exhibited a strong nucleolar staining pattern with high titers of IgG1 and IgG2a immunoglobulins (Table 3). A nucleolar green fluorescence was found in the cells of the tissue sections prepared from the mercury- but not saline-injected mice. It should be noted that such intranucleolar staining was absent in nucleoli of inbred mercury-treated mice.

In a purpose of better understanding of autoantigen specificity, we colocalized such nucleolar patterns recognized by FITC- conjugated anti-Ig antibodies with loci recognized by commercial antibodies to nucleolar protein fibrillarin. The colocalizing procedure showed the whole coincidence of regions containing immunoglobulins with sites of nucleolar protein fibrillarin localization (Fig. 8C, D, E), allowing us to offer the hypothesis about different capability of autoantibodies to penetrate the cells in inbred and outbred mice after $HgCl_2$ treatment.

These results demonstrate for the first time that injection of mercury into the genetically geterogenous outbred mice induced autoantibodies which are able to penetrate into the cells of certain organs and react with their corresponding nucleolar antigens *in vivo*.

4. Discussion

The main hallmark of mercury-induced autoimmunity in genetically susceptible mice is the production of ANoA against the 34 kDa nucleolar protein fibrillarin (Abedi-Valugerdi, 2008). Because lots of studies have demonstrated that only homozygous mouse strains with susceptible H-2 genotypes are able to produce ANoA after mercury treatment, we next performed this work to test the ability of such heavy metal to induce ANoA production and IC deposition in heterozygous mouse population.

As demonstrated in Table 1 , 6 weeks of $HgCl_2$ treatment induced the ANoA production in both inbred and outbred mice in variable titers, whereas control saline-treated mice did not show any ANoA production. Moreover, it should be noted that magnitude of mercury-induced ANoA in outbred mice was even higher than that induced in inbred mice.

Another characteristic feature of HgIA we tested was the deposition of immune complexes in the kidney. According to literature reports, revealing IC in different parts of the kidney in autoimmune animals is usually done in two basic ways: on air-dried and acetone-fixed cryosections or on formaldehyde-fixed and paraffin-embedded sections (Chowdhury et al., 2005; Gobe & Nikolic-Paterson, 2005). Each of these procedures has its own advantages and disadvantages. Immunofluorescence (IF) staining of renal biopsies for the deposition of immunoglobulins and complement components is often the primary approach for a differential diagnosis of glomerular disease. However, a limitation of such IF applications is that they require frozen sections, which can suffer a loss of structural integrity during the process of tissue freezing (Gobe & Nikolic-Paterson, 2005). On the other hand, the problem with formaldehyde-fixed and paraffin-embedded sections is that tissue antigens are often denatured or masked (Chowdhury et al., 2005).

Thus, in the present work we tried to combine these approaches by fixing renal cryosections with formaldehyde under standard conditions along with routine clinical air-drying or acetone-fixing. This allowed us to use the highly informative IF method.

We showed that treatment of the cryosections with organic fixatives (acetone and methanol) led to appreciable damage of the renal tissue, but did not interfere with revealing IC in kidneys. These observations correlate well with scanning electron microscopy data, which showed damage to the plasma membrane of cells by fixing with acetone and methanol (Hoetelmans et al., 2001). We do not favor the air-drying of sections; despite its simplicity, preservation of cells on such cryosections was poor. Thus, the best choice of fixative for cell preservation was paraformaldehyde; it also appeared to be best in the context of information in that using it, we found out IC in renal proximal tubules in kidneys in all groups of mice. To our knowledge, there is no previous report on the presence of IC in proximal renal tubes.

In view of the fact that treatment with the organic fixatives leads to dehydration of the tissue, removing many water-soluble intracellular proteins, we suggest that the IC in proximal renal tubules consist largely of water-soluble complexes. Further, it does not seem strange that only the proximal tubules contained IC, while the distal tubules lacked them. It is known that molecules as big as immunoglobulins can be reabsorbed only in the proximal tubules, while the main function of the distal parts of the nephron is reabsorbtion of electrolytes.

In contrast to deposits in proximal renal tubules, glomerular IC in kidneys of autoimmune animals have been mentioned in the literature many times (Abedi-Valugerdi et al., 1997; Bigazzi, 1999; Havarinasab et al., 2008; Hultman et al., 1987, 1992, 1993; Kono et al., 2001). However, there is a question as to where (in mesangial cells and/or on their surface) they are located. Our results, merging areas with IC and DAPI staining by epifluorescence and confocal microscopy, suggest that, at least, the major part of deposits is localized inside the cells. Nevertheless, we cannot exclude the possibility that some part of the deposits is situated on the cell surface. For a definitive conclusion about the localization of glomerular IC, a study using electron IHC is necessary.

In addition to renal IC, we have found immune deposits in increased titers in liver hepatocytes and the white pulp spleen cells, suggesting in favor that HgIA is a comprehensive process involving many organs.

Furthermore, the development of HgIA is accompanied by the occurrence of IC in blood vessel walls (Hultman et al., 1993). It was recently shown that the dye Col-F binds selectively with the collagen and elastin fibrils in coats and elastic membranes of blood vessels in native tissues. However, the possibility of using Col-F in combination with the IHC analysis has not been reported previously. Thus, an original protocol for the simultaneous staining of collagen and elastin fibrils and immunolabeling of immune deposits was developed. Our observations, based on specific staining of collagen and elastic fibers as a part of vessel walls with the dye Col-F, allowed us to localize the IC in both the

endothelial zone and across the whole width of the vessel walls in kidney, liver and spleen of mercury-treated mice. Additionally, revealing fibers using Col-F allowed us to visualize even the small vessels that were poorly identified with phase contrast because of elastic membrane thinning and luminal occlusion after tissue freezing.

According to our results, IC were present not only in organs of autoimmune mice, but also in the glomeruli, renal proximal tubules, hepatocytes and in the cells of spleen lymphoid follicles of control animals, although at a much lower level. This does not seem strange, because it is well-known in medicine that autoantibodies are found not only in the blood of autoimmune patients, but also in healthy individuals. In particular, sera from healthy people are capable of staining different cells (e.g., Hep-2) in an IIF reaction (Koelsch et al., 2007). However, the concentration (titer) of such antibodies is at least an order of magnitude less than in autoimmune patients. So, it seems possible that such antibodies in renal glomeruli, hepatocytes and proximal tubules could be derived from blood by filtration and primary urine by reabsorbtion, respectively, and become deposited in the cells. And the presence of immunoglobulins in cells of lymphoid follicles in the spleen is a sign of the normal functioning of the immune system.

Our investigation shows that immune complex deposits can differ not only in quantity, but also in composition. The C3 component of the complement system was seen as part of the renal IC only in the glomeruli and blood vessel walls, but not in the proximal tubules. In contrast with earlier studies (Hultman et al., 1987), the present investigation showed that some areas of the glomeruli containing C3 lacked Ig. Reasons for these features of IC composition can be a subject of future research. For these purposes, use of a laser microdissector with subsequent mass-spectrometric analysis of the cut areas may be useful.

Moreover, as described in the Results, renal IC consist of not only different classes but also different immunoglobulin isotypes. We demonstrated that glomerular IC include IgG, IgM, and, possibly, IgA immunoglobulins, while the vessel wall and proximal tubules contain almost no IgM.

More interesting is the occurrence of IgG immunoglobulin isotypes in the immune complexes. Results from initial studies suggested that HgIA in mice is mediated by a T helper type 2 (Th2) response (i.e., polyclonal B cell activation with hyper-IgE and -IgG1 production) (Abedi-Valugerdi, 2008; Gillespie et al,. 1995, 1996; Goldman et al., 1991; Ochel et al., 1991). However, further studies have revealed that the development of mercury-induced autoimmune manifestations cannot be explained simply by Th2-based immune response (Abedi-Valugerdi, 2008). In particular, IFN-ɣ, a key cytokine produced by activated T helper type 1 (Th1) cells, induces IgG2a response in the mouse and is absolutely required for the induction of ANoA production in this autoimmune condition (Abedi-Valugerdi 2008; Kono et al., 1998). Our results are consistent with this. Despite the Th2-dependent appearing of germinal centers in spleen after HgCl$_2$-treatment and the prevalence of the Th2-mediated IgG1 isotype as a component of IC, we also saw some IgG2a in the deposits, arguing in favor of Th1 activation too.

With all that the most important result of our research is the discovery of the ability of $HgCl_2$-induced ANoA to transverse the plasma and nuclear membrane of living cells and translocate to the nucleoli of different cells in outbred mice in vivo. We found that mercury-induced ANoA penetrated into the cells of certain organs (kidney, liver and spleen) and colocalized with special nucleolar protein fibrillarin - the major autoantigen in HgIA. This fact once again place outbred mice in close quarters with humans. As is well known from the literature, the autoantibodies from SLE patients are able to penetrate into the nuclei of cells in certain organs (Foster et al., 1994; Vlahakos et al., 1992; Yanase et al., 1997; Zack et al., 1996). It is likely that mercury-induced ANoA contain basic amino acid-rich sequences similar to those seen in anti-DNA autoantibodies derived from lupus-prone mice, allowing them to penetrate into the cell nuclei (Abedi-Valugerdi et al., 1999). Several studies have shown that penetrating autoantibodies cause cellular dysfunction after entering the cell and reacting with their intracellular antigens (Abedi-Valugerdi et al., 1999; Koscec et al., 1997; Reichlin, 1995). Therefore, it has been suggested that these antibodies might have pathogenic roles. However, our results do not confirm this, at least, at the beginning of the development of such mercury-induced autoimmune response. Formation of IC in different parts of the kidney was not accompanied by visible destruction or cell death, at least as evidenced by the TUNEL assay. The article of Abedi-Valugerdi et al. (1999) offers two possible explanations of this fact. First of all, the main target for mercury-induced ANoA is fibrillarin, which is known to be associated with snRNAs. In mammals the exact function of fibrillarin is not known, but it has been suggested that this nucleolar protein possibly participates in ribosomal biosynthesis. Based on this suggestion, it is likely that fibrillarin does not have a crucial role in the DNA synthesis and interfering with its function/structure by ANoA would not impair the cell proliferation. Second, since besides fibrillarin, several other nucleolar proteins (nucleolin, Surf-6, etc.) are also present in the mammalian nucleoli, it is likely that if fibrillarin is required for DNA synthesis and if binding of ANoA to fibrillarin impairs it's function, other nucleoproteins will take over fibrillarin's function. Since it has been suggested that fibrillarin is involved in the synthesis of ribosomal RNA, further studies are needed to test if nucleolar localization of ANoA would affect other cell functions such as protein synthesis. But, nevertheless, we cannot exclude the third possibility that destructive alterations could appear in later stages of disease development, because it is known that renal failure is one of the negative features associated with human autoimmune diseases (Tormey et al., 2001).

5. Conclusion

Thus, in our work we have shown that $HgCl_2$ induce very strong autoimmune process both in inbred and outbred mice, accompanied by ANoA production and heavy IC deposition. We have described novel localization and composition of such immune deposits in different organs. Also, we have come to conclusion about the higher penetrating capability of autoantibodies in outbred mice as compared with inbred mice. So, we have discovered that genetically heterogenous outbred CFW mice produce the same reaction on standard $HgCl_2$ treatment (1.6 mg/kg twice a week) as inbred SJL/J mice previously described to be most susceptible.

Our data thoroughly confirm and continue the findings of Dr. Abedi-Valugerdi suggesting that certain environmental factors, without requiring the presence of specific susceptibility genes, can induce some autoimmune manifestations in members of a genetically heterogenous population.

We think that outbred mice with $HgCl_2$-induced autoimmunity may also be used for testing of immunosuppressive drugs because they better reflect the human population then homozygous inbred mice. Thus, the present study could be very useful for further understanding, prediction and therapy of human systemic autoimmune diseases, in particular developing after the regular exposure of mercury compounds.

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