Chapter 2

Minor and Trace Elements in Whole Blood, Tissues, Proteins and Immunoglobulins of Mammals

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Additional information is available at the end of the chapter

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Abstract

Microelements play different important roles in many physiological processes in all biological systems in both normal physiological and pathological conditions. They take part in the transport of nutrients and gases, support temperature, acid-base balance, homeostasis of the human organisms, maternal and child mental health, the functioning of enzymes, protein and DNA syntheses, cytoskeleton activation, etc. We have performed simultaneous determination of a number of minor and trace elements in whole blood and tissues of mammals by two-jet plasma atomic emission spectrometry (TJP-AES). TJP-AES allows direct analysis of powders without wet acid digestion and can be used for analysis of both large and small amount of the sample, which is important for biomedical investigations with humans and experimental animals. In addition, a content of different elements in preparations of human immunoglobulins was estimated by TJP-AES as well as using different physicochemical methods, the functional role of metal ions in antibodies functioning was analyzed. The analysis of the relative activity of antibodies with catalytic activity (abzymes) in the hydrolysis of DNA, RNA, proteins, peptides and oxidation-reduction reactions and the role of metal ions in the catalysis of these reactions by abzymes were carried out.

Keywords: two-jet plasma atomic emission spectrometry analysis, minor and trace elements, functional role of metals

1. Introduction

Minor and trace elements including metals play important roles in many systems, normal and pathologic processes. Nowadays, there are many methods for elemental analysis of different biological samples. Atomic absorption spectrometry (AAS), inductively coupled plasma atomic

emission spectrometry (ICP-AES) and mass spectrometry (ICP-MS) are usually used for analysis of blood and animal tissues [1, 2]. Generally, these methods require matrix destruction with concentrated acids. Using microwave-assisted wet acid digestion with temperature control and elevated pressure allows reducing the time of sample digestion and risk of element losses. For direct ICP-MS analysis of whole blood and serum, dilution with alkaline solutions containing EDTA, ammonia and Triton X-100 was also employed to lyse the blood cells and prevent blood clotting [3, 4]. To improve the analytical capabilities of the methods applied, the new analytical techniques and reagents are being developed. A collision/reaction cell technology allowed the removal of polyatomic interferences and extended the capabilities of ICP-MS for trace element determination [5]. To decrease limits of detection (LODs) of trace elements, different ways of their preconcentration were offered. For determining the low concentration of Pb in blood serum by flame AAS, Barbosa with coauthors [6] used oxidized carbon nanotubes covered with bovine serum albumin layers; preconcentration was performed in untreated blood serum and allowed getting LOD of Pb at the level of 2 μg/L. Mortada with coauthors [7] suggested hydroxyapatite nanorods prepared from recycled eggshell for solid phase extraction of Pb, Cu and Zn from solutions of different biological samples followed by AAS analysis. In spite of undeniable progress in developing the above-mentioned methods, the analysis of biological samples using solid sampling is very attractive as the analytical procedure is simple and risk of contamination and analyte losses is improbable. To adapt the above methods for direct analysis of solid biological samples, laser ablation (LA) and electrothermal vaporization (ETV) were applied. LA-ICP-MS has got significant attention over the last decade for the analysis of biological samples [8]. It has been mainly applied to produce images of element distributions in human and animal tissues, which is of great scientific interest [9]. The main challenge of facing the LA-ICP-MS application is fully quantitative analysis requiring complex strategies for producing reliable calibrating materials. Lack of certified reference materials (CRMs) with different biological matrices and complications of preparing matrix-matched calibration samples [10] often make it difficult to carry out quantitative multi-elemental analysis of different biological tissues. This problem is well known for ETV-ICP-AES, X-ray fluorescence and laser-induced breakdown spectrometry (LIBS).

In recent times, LIBS has been applied as a screening tool for trace element bio-imaging in human and animal organs. To detect Wilson's disease, the study of Cu distribution in human liver was carried out [11]. A low-cost approach allows the quick detection of pathological accumulation of Cu in the affected organs. The LIBS mapping of the mice kidney slices after injection of a solution containing Gd-based nanoparticles was performed [12]. Each of the above methods has both advantages and disadvantages, and the choice of the analytical method depends on the sample nature, analytical tasks and availability of appropriate device and calibration samples. In the present work, two-jet plasma atomic emission spectrometry (TJP-AES) was used for analysis of different biological samples.

2. Two-jet plasma atomic emission spectrometry

2.1. A two-jet plasmatron

The TJP was developed by Zheenbaev and Engel'sht in the USSR (Kyrgyzstan Institute of Physics) in the mid-1970s [13]. It is a direct current (dc) plasma that differs from dc plasmas described [14, 15]

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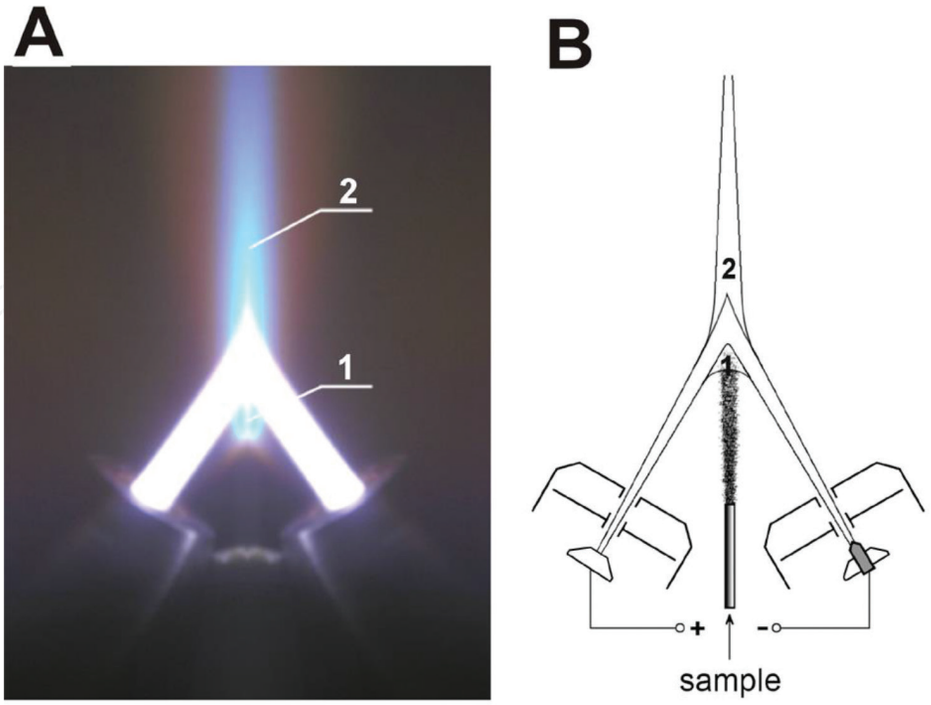

Figure 1. (A) Plasma torch; (B) electrode unit and analytical regions of the plasma flow: 1, before the jet confluence; 2, after the jet confluence.

and an ICP by a high power, which allows analysis of powdered samples without sample dissolution. Although the TJP appeared approximately at the same time as an ICP, TJP-AES was not generally recognized since only a few copies of the plasmatron were produced; it has not been modernized for decades. Nowadays, TJP-AES is experiencing a new stage in its development because a new modern plasmatron was designed at "VMK-Optoelektronika" (Russia). A photograph of the plasma torch and scheme of electrode unit are presented in **Figure 1**.

Argon plasma jets are generated in non-consumable electrodes (copper anode and tungsten cathode); they join at the output to form plasma discharge. The argon consumption does not usually exceed 5 l/min. The power supply of the plasma generator, gas flow and automatic sample introduction systems are computer controlled. A TJP power can be fixed from 5 to 12 kW by varying the current strength in the range of 40–100 A. Current fluctuation does not exceed 1%. A new plasmatron is equipped with a concave diffraction grating (2400 lines/mm) and two multicrystal photodiode arrays allowing spectra to be measured in two spectral ranges: 190–350 and 390–450 nm.

To transfer powders into the plasma, a powder introduction device was developed. The sample is placed in a Plexiglas beaker, inserted into the device and roiled with blast waves produced by a spark between zirconium electrodes over the surface of the powder. An aerosol obtained is delivered into the plasma with a carrier gas. The device allows the introduction of both small and large amount of the samples (5–500 mg).

2.2. Analytical regions

There are two analytical regions in the TJP – before and after the jet confluence (**Figure 1**). The region of the confluence is not used for analysis due to high background emission. A study of behavior of a wide range of elements in the plasma has shown that the highest ratio of the

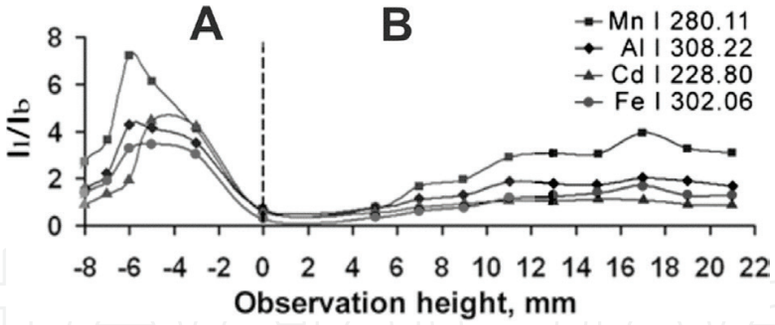

Figure 2. I_l/I_b distribution along the plasma flow for Mn I 280.11, Al I 308.22; Cd I 228.80 and Fe I 302.06 lines: region (A) before the jet confluence, (B) after the jet confluence; 0 – point in the region of the confluence.

analytical line intensity to the background one (I_l/I_b) realizes in the region before the jet confluence both for atomic and ionic lines. As an example, the distribution of I_l/I_b for analytical lines Mn I 280.11, Al I 308.22; Cd I 228.80 and Fe I 302.06 along the plasma flow is shown in **Figure 2**.

Analytical lines of Ag and Zn registered in the region before and after the jet confluence are shown in **Figure 3**.

As it is seen, the analytical signals are at the level of background fluctuations in the region after the jet confluence, but they are considerably higher than background in the region before one, which testifies to better LODs of elements in this region. One of the problems arising in the TJP-AES analysis of powdered samples is their incomplete evaporation which can lead to a systematic underestimation of the analysis results. The short residence time of the sample in the plasma is one of the reasons for the partial sample evaporation. In addition, evaporation efficiency depends on chemical composition, structure and particle size of powdered samples [17]. To demonstrate evaporation efficiency in the analytical zones of the TJP, silicon carbide (SiC) powders with an average particle size of 1, 3, 7.5, 17, 22 and 36 μm were used. SiC is a heat-resistant material having a high hardness close to the hardness of diamond. SiC evaporation was controlled by the intensity of weak Si I 212.30 line. To avoid signal self-absorption, the powders were diluted with graphite 100 times. The dependence of Si I 212.30 line intensity on the particle size is given in **Figure 4**.

The different behavior of silicon line is observed in the regions investigated. In the region before the jet confluence, there is an increase in the silicon line intensity along with a decrease in the particle size, the smaller particle size and the better evaporation efficiency. However, the complete sample evaporation does not occur even at a particle size of 3 μm. In the region after the jet confluence, the maximum intensity is achieved even at a particle size of 17 μm. The decrease in intensity at smaller particles seems to occur due to the introduction of light particles into the region after the confluence is complicated by a resistance of the consistent plasma jets. Nevertheless, the effect of particle size on evaporation efficiency is considerably weaker in the region after the jet confluence. Thus, the region before the jet confluence provides lower detection limits of elements than the region after one, but evaporation efficiency is better in the region after the jet confluence. Therefore, the choice of the analytical region depends on an analytical task and sample nature.

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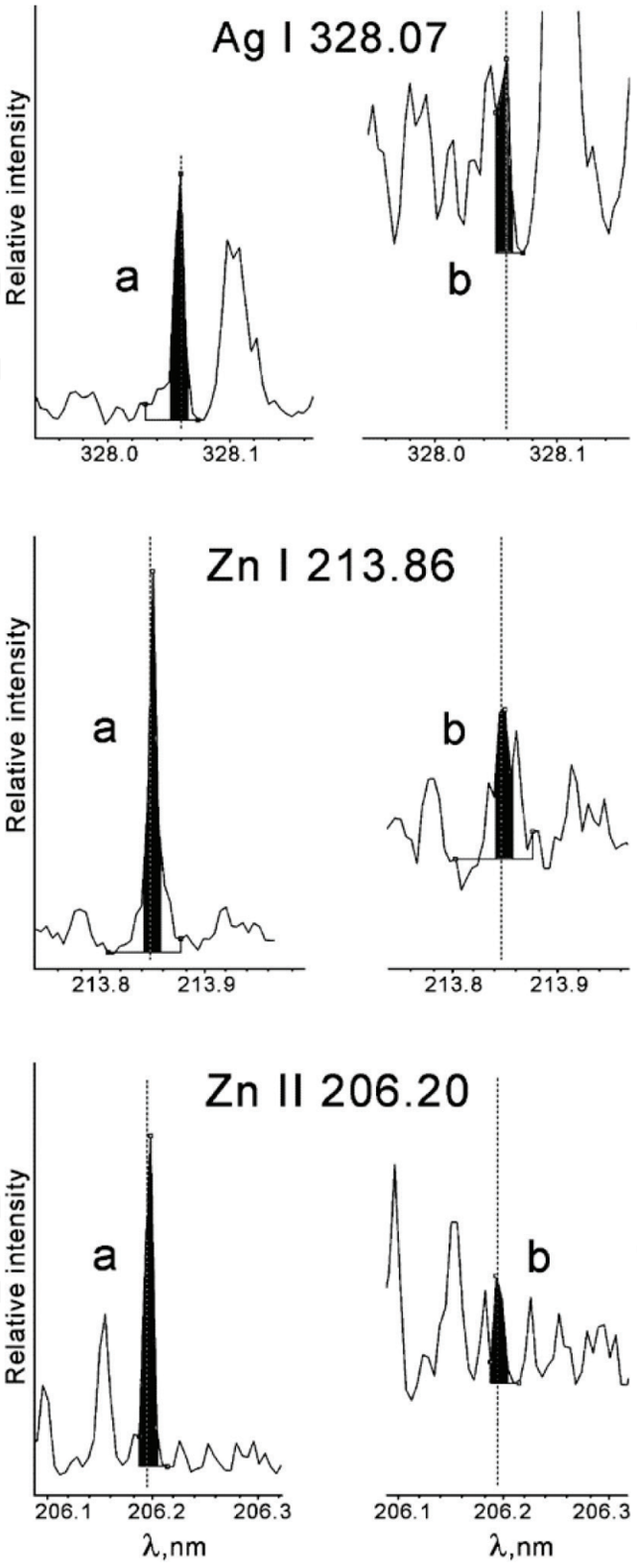

Figure 3. Analytical lines Ag I 328.07 (0.05 μg/g), Zn I 213.86 (0.5 μg/g) and Zn II 206.20 (1 μg/g) registered in the region (a) before the jet confluence and (b) after the jet confluence [16].

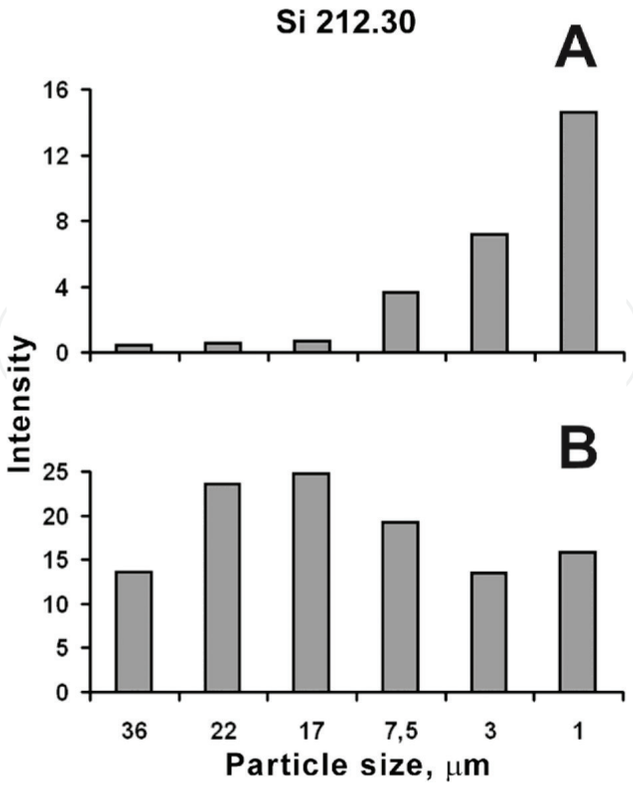

Figure 4. Dependence of Si I 212.30 line intensity on the particle size of SiC in the region (A) before the jet confluence (B) after the jet confluence.

2.3. Excitation mechanisms

To solve the problems appearing in the analysis, it is important to have an idea about the processes occurring in the plasma. The probable mechanisms of an atom and ion excitation in the TJP were investigated [16, 18]. The Boltzmann distribution of excited energy levels for Fe atoms and singly ionized ions was found to take place in both analytical regions, which indicates the predominant excitation of atoms and ions by electron impact. Excitation temperature of Fe atoms and ions, T_{atom} and T_{ion}, respectively, was measured along the plasma flow. In the optimal observation zone of the region before the jet confluence T_{atom} = 6000 K and T_{ion} = 7900 K, and in the observation zone of the region after one T_{atom} = 7060 K and T_{ion} = 8050 K. For atomic and ionic lines, the temperature deviation did not exceed 100 K in the optimal observation zones and was about 250 K near the jet confluence. The considerable difference in T_{atom} and T_{ion} points at the departure from local thermodynamic equilibrium (LTE) in the plasma. The difference is 1900 K for the region before the jet confluence and 990 K for the region after one, which indicates that the region before the jet confluence is more non-equilibrium than the region after one. The disturbance of LTE in the plasma was shown to be due to metastable argon participation in atom ionization.

3. Application of TJP-AES

Originally, the TJP was intended for direct analysis of sparingly soluble geological samples [19], which considerably reduced the analysis time and element losses as compared with wet acid digestion. The spectra were registered in the analytical region after the jet confluence

(**Figure 1**), and calibration samples similar to the analyzed ones were used. The region before the jet confluence was not practically used for analysis.

3.1. Analysis of high purity substances

The region before the jet confluence turned out to be suitable for analysis of high purity substances both by direct technique and after matrix separation. The TJP-AES techniques for analysis of gallium [20], indium and indium oxide [21] and tellurium dioxide [22] were developed. The direct techniques allow determination of about 30 elements using appropriate dilution of the sample with a spectroscopic buffer (graphite powder containing 15 wt.% NaCl) and unified calibration samples based on graphite powder with addition of 15 wt.% NaCl. As it was shown earlier, NaCl addition increases analytical line intensities and suppresses effects of a mineral matrix [23]. CRMs of graphite powder with different combinations of impurities are commercially available (Ural Federal University, Russia); in addition, preparing the reference sample with given element concentration in graphite is not a difficult task. Analysis of the above substances was carried out at the optimal conditions chosen for multi-elemental analysis of graphite powder (**Table 1**). Calibration curves (lgI-lgC) obtained for Cd and Hg in graphite powder are presented in **Figure 5**.

A degree of sample dilution depends on the sample nature; a fourfold dilution is needed for analysis of indium oxide and gallium, and a twofold dilution is quite enough for analysis of tellurium dioxide. The preconcentration of impurities in gallium and indium was accomplished by matrix separation in the form of chlorides; tellurium dioxide was previously reduced by hydrogen to metal, and the preconcentration was performed by vacuum distillation of tellurium. The impurity concentrates contained a high concentration of matrix elements since incomplete matrix separation was applied to avoid the loss of a number of important impurities; they were analyzed by the same way as in the direct techniques. LODs of elements were at the level of 0.01–1 and 0.001–0.1 μg/g for direct analysis and after matrix separation, respectively. The possibility of analysis of such a different substances using unified calibration samples points at comparatively weak matrix affects this excitation source. For comparison, using such an approach for a dc arc with sample evaporation from a crater of graphite electrode requires a 100-, 50-, and 25-fold dilution of gallium, indium and tellurium oxides, respectively, which lead to worsening LODs of elements by more than an order of magnitude. Recently, the similar approach was used for analysis of different soils [24]. In spite of their complex and variable matrix composition, TJP-AES allowed direct determination of

Parameter	Value
Current strength	85 A
Voltage	120 V
Plasma gas flow	4 l/min
Carrier gas flow	0.85 l/min
Angle between the jets	60°
Observation zone	4–5 mm lower than the point of the confluence

Table 1. Working conditions of the two-jet plasma.

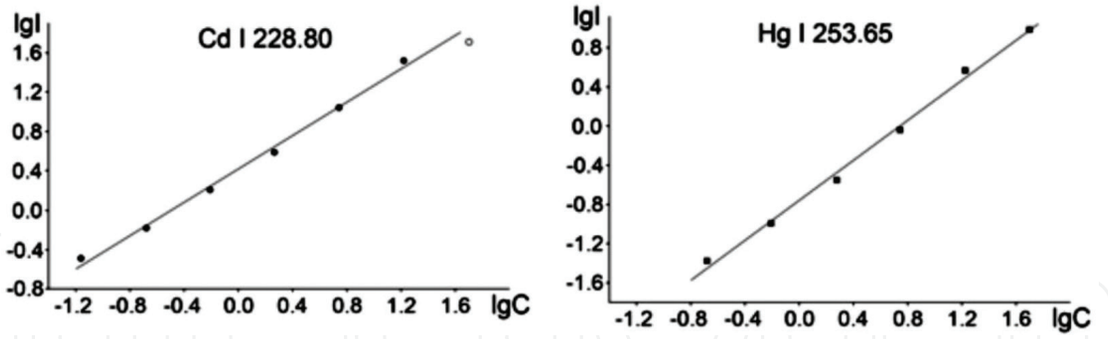

Figure 5. Calibration curves for analytical lines Cd I 228.80 and Hg I 253.65 (C μg/g) [24].

As, B, Cd, Cu, Hg and P after a twofold and Be, Co, Cr, Ga, Nb, Pb and Zn after a 10-fold dilution with a spectroscopic buffer.

3.2. Analysis of biological samples

Solid sampling, with little or no chemical pretreatment, in the analysis of biological samples seems very attractive. The possibility of TJP-AES for direct analysis of biological samples using the same unified approach as for inorganic materials was investigated. First, starch was used to study the organic matrix influence on analytical signals of elements in the TJP [25]. It was found that the presence of 10 wt.% starch in graphite powder with introduced impurities did not affect analytical line intensities of elements while the decrease in intensities by a factor 2–5 was observed in a graphite dc arc. In the TJP, the effect was not observed even in the presence of 20 wt.% starch for many atomic lines of elements. The considerable decrease in intensities in a dc arc is due to a vigorous reaction of starch with air oxygen in an arc discharge with the release of gaseous products, which results in decreasing the residence time of the sample in the plasma. Although the TJP is an open system too, the oxidative reaction occurs less violently than in a dc arc since carrier argon partially displaces air from the excitation zone, and the sample is gradually introduced into the plasma. In addition, gaseous products seem to be retained in the excitation zone by argon flows. The experiments with starch gave hope to get positive results for more complex organic matrices.

3.2.1. Analysis of animal organs

The unified approach mentioned above was tested for animal organs, dried and finely powdered. Dry animal organs contain more than 50 wt.% proteins as well as fats, carbohydrates and others. The effect of such a complex matrix on the analytical signal of elements was studied by the analysis of a spiked sample based on graphite with the addition of 15 wt.% NaCl and 10 wt.% rat liver; concentration of elements introduced was 2.5 μg/g [26]. The analysis was carried out using calibration samples based on graphite powder with addition 15 wt.% NaCl; the spectra were observed in the region before the jet confluence. The analysis results are given in **Table 2**; the satisfactory recoveries were obtained for all investigated elements. Since the liver contained Co, Mn, Mo and Zn, the blank sample was prepared to estimate correctly the recovery of these elements. On the basis of the results obtained, a 10-fold dilution

Element	λ (nm)	Concentrations of elements		Recovery (%)
		Added (μg/g)	Found (μg/g)[a]	
Ag	338.29	2.5	2.4 ± 0.6	96
Ba	233.53	2.5	2.5 ± 1.0	100
Be	265.06	2.5	2.3 ± 0.8	92
Bi	306.77	2.5	2.6 ± 0.8	104
Cd	228.80	2.5	2.5 ± 0.3	100
Co	345.35	–	0.26 ± 0.8[b]	–
		2.5	2.8 ± 0.6	102
Cr	283.56	2.5	2.8 ± 1.4	112
Ga	294.36	2.5	2.4 ± 0.4	96
Hg	253.65	2.5	2.6 ± 0.6	104
Mn	280.11	–	0.80 ± 0.15[b]	–
		2.5	3.5 ± 0.6	108
Mo	317.03	–	0.27 ± 0.10[b]	–
		2.5	2.5 ± 0.3	89
Ni	305.08	2.5	2.3 ± 0.7	92
Pb	283.31	2.5	2.5 ± 0.4	100
Sb	231.15	2.5	2.5 ± 0.7	100
Sn	284.00	2.5	2.3 ± 0.5	92
Zn	213.85	–	6.8 ± 1.7[b]	100
		2.5	9.3 ± 2.0	

[a]Mean ± (95% confidence interval), n = 4.

[b]Value obtained without spike addition.

Table 2. Determination of elements in a spiked graphite powder containing 10% rat liver [26].

of powdered animal organs with buffer and calibration based on graphite powder were suggested for direct analysis of animal organs. To validate the technique, the results of direct analysis of bovine liver were compared with the results obtained after sample carbonization (500°C, 5 min) and autoclave digestion in a mixture of nitric acid and hydrogen peroxide [25]. The carbonized sample was analyzed at the conditions chosen for direct analysis. The solution obtained after acid digestion evaporated on graphite powder, diluted with buffer and analyzed by the same way. As it is seen from **Table 3**, the results of Al, Ca, Cu, Fe, Mg, Mn, Mo, P, Si and Zn satisfactory agree with each other. Only for Fe, Mn and Mo, the results obtained after autoclave digestion are lower than the results of the direct technique, which is likely to be due to their partial loss. The LODs of elements provided by the direct technique are at the level 0.1–10 μg/g, and they are lower by approximately one order of magnitude after carbonization. The use of the carbonization procedure allowed determining the low concentrations of Ag, Cd, Co, Cr, Pb and Ni in the liver.

B	249.77	6.2 ± 2.0	2.7 ± 0.5	5.5 ± 1.0
Ca	317.93	130 ± 20	110 ± 30	130 ± 40
Cd	228.80	<0.4	0.17 ± 0.03	0.25 ± 0.10
Co	345.35	<1	0.31 ± 0.05	—
Cu	324.75	12 ± 2	12 ± 4	10 ± 0.2
Cr	283.56	<1	0.2 ± 0.04	0.24 ± 0.06
Fe	302.06	250 ± 40	210 ± 70	150 ± 30
Mg	277.98	470 ± 80	460 ± 120	420 ± 90
Mn	279.83	16 ± 2	13 ± 3	9.5 ± 2.4
Mo	317.03	18 ± 3	20 ± 5	9.0 ± 2.3
Ni	305.08	<1	0.6 ± 0.1	0.77 ± 0.25
P, wt.%	214.91	0.99 ± 0.08	0.85 ± 0.25	0.76 ± 0.11
Pb	283.31	<1	0.40 ± 0.07	0.40 ±0.11
Si	288.16	22 ± 8	31 ± 14	22 ± 11
Zn	213.86	62 ± 15	55 ± 10	60 ± 8

[a]95% confidence interval.

Table 3. Results of the TJP-AES analysis of bovine liver (μg/g) [25].

The technique suggested is very suitable for analysis of dry internals such as liver, kidney and spleen which are easily ground in a Plexiglas mortar to a powder with the particle size of 20–30 μm. However, the direct analysis of bovine and pork muscle CRMs and rat brain was found to provide the understated results [26]. These tissues are more thermostable than liver and have the particles of more than 100 μm. Flexible fibers of muscles and plastic consistence of brain make it difficult to obtain a powder with small particles. Incomplete evaporation of the samples is the most probable reason for the result underestimations. This problem was overcome by decreasing the consumption of a carrier gas, which increases the residence time of the sample in the excitation zone and concentration of air oxygen participating in organic matrix decomposition. It should be noted that the carbonization conditions are not all-purpose and depend on a kind of tissues. For brain and muscle tissues, the time of carbonization was increased up to 30 min. Thus, in spite of the unified approach to the analysis of organs, peculiarities of different tissues should be taken into account. For direct analysis, 5–10 mg of powdered sample is quite enough; for carbonization procedure, 50–100 mg of the sample is needed. Note that the ICP-AES and ICP-MS techniques with wet acid digestion of organs usually require 100–250 mg of the sample which is not always available. The relative standard deviation of the analysis results of animal organs usually is in the range of 3–12%.

3.2.2. Analysis of whole blood

The problem of availability of biological samples in ample quantity is particularly acute in experiments with living experimental animals (such as mice or rats). Blood is the main subject

Element	λ (nm)	Human blood			Rat blood		
		Direct analysis	Carbonizing	Autoclave digestion	Direct analysis	Carbonizing	Autoclave digestion
Fe	296.67	2300 ± 200[a]	2200 ± 160	2200 ± 200	2600 ± 200	2400 ± 170	2500 ± 200
P	214.91	1700 ± 130	1600 ± 120	1500 ± 110	1400 ± 200	1400 ± 100	1300 ± 150
Ca	317.93	320 ± 20	340 ± 30	300 ± 20	240 ± 30	250 ± 20	280 ± 30
Mg	277.98	130 ± 15	150 ± 20	130 ± 10	120 ± 15	140 ± 15	150 ± 20
Zn	213.86	28 ± 1.6	26 ± 1.4	29 ± 1.7	29 ± 2.0	32 ± 1.5	32 ± 1.4
Cu	324.75	6.6 ± 0.5	6.2 ± 0.4	5.8 ± 0.4	3.6 ± 0.3	3.5 ± 0.3	3.3 ± 0.2

[a]95% confidence interval, n = 4.

Table 4. Concentration of elements in the freeze-dried blood samples (μg/g) [27].

of investigation for living organisms. Whole blood quickly changes over time due to fast clotting, which troubles the sample cutting. The effect of anticoagulants also lasts a limited time. Therefore, for continuous biomedical experiments, freeze-dried whole blood which can be kept for a long time at normal conditions is very convenient. For determining the main essential elements (Fe, P, Ca, Mg, Zn and Cu) in freeze-dried whole blood, the direct technique developed for animal organs was applied [27]. To confirm the possibility of such an approach for blood analysis, the direct analysis of CRM of freeze-dried bovine blood (IAEA A-13) was carried out, and good agreement of the results with the certified values was obtained. In addition, the results of analysis of human and rat freeze-dried whole blood obtained using the different sample preparation procedures were compared (**Table 4**). The direct technique results satisfactory agreed with the results obtained after carbonization (400°C, 15 min) and autoclave digestion, which confirms the possibility of the unified approach for blood analysis. Simple sample preparation (dilution with buffer) and the possibility of analysis of small amount samples, 5–10 mg of blood powder (approximately 20–50 μL liquid blood), are of practical importance. For analysis of whole liquid blood, blood aliquots evaporated on graphite powder under an IR lamp and then carbonized at 400°C for 15 min. The remainder was ground in a mortar and analyzed as in the direct analysis. The techniques suggested were validated for analysis of both freeze-dried and liquid blood serum and plasma.

3.2.3. Analysis of bone

Bone is a highly mineralized mobile tissue which accumulates inorganic substances and diffuses them as the need arises. It contains 25 and 65 wt.% organic and inorganic substances, respectively, and 10 wt.% water. Collagen and calcium hydroxyapatite $Ca_{10}(PO4)_6(OH)_2$ are the main components of bone. Some elements are predominantly in the organic phase, and others are in the mineral phase of bone. It was found that a fourfold dilution of dry powdered bone was quite enough for element determination by the unified direct technique [28]. The results of "added-found" experiment and comparison of the direct technique results with the results of ICP-AES after wet acid digestion of the sample validated the technique. However, underestimating the Ba, Mg, and Sr concentration was obtained. These elements are strongly

Element	λ (nm)	LOD (μg/g)
Ag	328.07	0.1
Bi	306.77	1.2
Cd	228.80	0.5
Co	345.35	1.2
Cr	283.56	0.3
Cu	324.75	0.2
Fe	296.68	2.0
Ga	294.36	0.3
Mn	260.57	0.5
Mo	313.26	0.7
Ni	305.08	0.6
Pb	283.31	1.1
Sn	284.00	0.8
Zn	213.86	1.0

Table 5. Limits of detection (LOD) of elements in bone [28].

bound with calcium hydroxyapatite, and its incomplete evaporation may lead to their understated concentrations. This effect took place even at a mean particle size of 30 μm. The strongest decrease in concentration was observed for Sr replacing Ca in hydroxyapatite. It is well known that strontium rachitis develops in the regions with a high content of radioactive Sr due to the formation of high concentration of strontium hydroxyapatite in bone, which results in the fragility of people and animal teeth and bones. Pretreatment of the samples with nitric acid followed by heating at 300°C or decrease of the consumption of carrier argon as in the case of brain and muscle tissues allowed getting valid results for Ba, Mg, and Sr. The satisfactory results obtained for other elements by direct technique point at their fractional volatilization from the particles in the plasma. These elements seem to be bound with the organic portion of bone or weakly bound with calcium hydroxyapatite. LODs of a number of elements in bone are given in **Table 5**.

Thus, on the example of different animal organs, whole blood and bone the possibility of TJP-AES for realizing the simple analytical techniques was shown. Solid sampling, unified calibration samples, the possibility of analysis of small amount samples are of great interest for experiments with different biological tissues.

4. Analysis of trace element changes in mice treated with $CoCl_2$

Transition-metal cobalt is an essential trace element required for vitamin B_{12} biosynthesis, enzyme activation, etc., but is toxic in high concentrations. We estimated the effect of cobalt

chloride ($CoCl_2$) on relative content of different metal ions in mouse plasma using TJP-AES and on the total protein content [29, 30]. Freeze-dried plasma (2–3 mg) was available for the TJP-AES analysis. On average the relative content of different elements in the plasma of 2-month-old mice balb/c (control group) decreased in the order: Ca > Mg > Si > Fe > Zn > C u ≥ Al ≥ B. The 60 days treatment of mice with $CoCl_2$ (daily dose 125 mg/kg) did not change appreciably the relative content (ReCo) Ca, Cu and Zn, while a 2.3-fold significant decrease in the ReCo of B and a significant increase in the content of Si (3.4-fold), Fe and Al (2.1-fold) and Mg (1.5-fold) was found (**Table 6**). The ReCo of Mo and Co for untreated mice was lower than test sensitivity. Mo in a detectable amount was determined only for two mice in the control group, but the plasmas of 9 out of 16 mice of analyzed group contained this metal. Cobalt treatment resulted in a 2.2-fold decrease in the concentration of total plasma protein and in 1.7-fold immunoglobulins. Clarification of the complex effects of Co^{2+} on its interactions *in vivo* with other trace elements is important for the explanation of cobalt toxicity and disturbances in homeostasis and physiological processes such as development, growth, weight gain, immunity, reproduction, etc. [29].

Homogeneous IgGs purified from sera of mice treated (t-IgGs) and non-treated (nt-IgGs) with $CoCl_2$ containing intrinsically bound metal ions hydrolyze DNA with very low activity and lose this activity in the presence of EDTA [30]. The average relative DNase activity (RAs) of nt-IgGs increased after addition of external metal ions in the following order: $Zn^{2+} < Ca^{2+} < Cu$

Chemical element	Control untreated mice (n = 19)		Mice treated with $CoCl_2$ (n = 16)		Ratio of (2) and (1)*	Significance (*P*)
	Range of values (μg/g)	Average value (μg/g) (value 1)	Range of values (μg/g)	Average value (μg/g) (value 2)		
Ca	540–1670	1099 ± 239	510–1670	1169 ± 288	1.17	0.53
Cu	6.0–20.5	10.1 ± 2.6	5.2–26.5	9.1 ± 2.7	0.99	0.46
Zn	14–53	24.2 ± 6.8	15–49	25.9 ± 5.9	1.18	0.56
B	1.2–6.7	5.8 ± 2.5	1.1–5.6	2.5 ± 0.79	0.47	3×10^{-4}
Mg	190–590	423 ± 102	610–850	625 ± 75	1.76	1.2×10^{-7}
Al	4–13	6.5 ± 2.9	8–25	13.8 ± 3.4	2.53	2.5×10^{-7}
Fe	40–130	69 ± 17.0	72–270	147 ± 50	2.53	2.0×10^{-7}
Si	50–190	101 ± 38	200–520	342 ± 92	4.07	1.0×10^{-9}
Mo	~0	~0	0–9	2.3 ± 2.2		—
Co	~0	~0	7.2–37	16.8 ± 6.5		—
Total protein, mg protein/ml of plasma						
Protein	9.0–16.6	13.7 ± 1.9	2.0–7.7	6.2 ± 1.9		

*For each mouse, the mean of three repeats is used.

Table 6. Relative content of different chemical elements and total protein in the freeze-dried blood plasma of control mice and animals treated with $CoCl_2$ [29].

$^{2+}$ < Fe^{2+} < Mn^{2+} < Mg^{2+} < Co^{2+} < Ni^{2+}. Interestingly, t-IgGs showed lower activity than nt-IgGs in the absence of external metal ions (2.7-fold) as well as in the presence of Cu^{2+} (9.5-fold), Co^{2+} (5.6-fold), Zn^{2+} (5.1-fold), Mg^{2+} (4.1-fold), Ca^{2+} (3.0-fold) and Fe^{2+} (1.3-fold). But t-IgGs were more active than nt-IgGs in the presence of Ni^{2+} (1.4-fold) and especially Mn^{2+} (2.2-fold), which are the best activators of t-IgGs. These data may be useful for an understanding of Co^{2+} toxicity, its effect on a change of metal-dependent specificity of mouse abzymes [30].

5. Abzymes with oxidation-reduction activities

First, we have estimated the content of metals in the lyophilized plasmas of healthy Wistar rats (**Table 7**) by the TJP-AES method [31]. The relative amount of metals in the rat plasma decreased in the order: Ca > Mg > Fe > Cu ≥ Zn > Al ≥ Sr. > Ti ≥ Mo ≥ Mn (**Table 7**).

Metal	Relative content (μg/g)*									Average value (μg/g)
	Rat number									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	
Ca	1700	1700	**1200**	1500	1700	1300	1200	1700	**2000**	1556 ± 274***
Mg	440	420	280	380	360	310	300	360	410	362 ± 56
Fe	**14****	120	90	110	80	110	**80**	100	110	104 ± 19
Cu	29	25	**22**	24	34	22	25	**39**	33	28.1 ± 6.0
Zn	26	44	**22**	25	**28**	25	26	25	26	27.4 ± 6.4
Al	6.0	**15**	7.0	13	6.0	5.0	5.0	**4.0**	7.0	7.6 ± 3.8
Sr	5.5	6.2	5.3	6.2	8.4	**5.0**	6.2	6.5	**8.4**	6.4 ± 1.2
Ti	**5.0**	3.0	3.0	3.0	3.0	4.0	**3.0**	4.0	4.0	3.6 ± 0.7
Mo	2.3	1.5	**1.1**	1.8	3.3	2.0	2.0	**3.4**	2.7	2.2 ± 0.8
Mn	1.2	**1.6**	1.1	**1.1**	1.3	1.4	1.2	1.4	1.3	1.3 ± 0.2
Pb	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0	<2.0
Cr	1.0	≤1.0$^{\Omega_1}$	≤1.0	≤1.0	≤1.0	**≤1.0**	≤1.0	≤2.0	**≤6.0**	≤1.7 ± 1.6
Ni	≤1.0-	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0	≤1.0
Co	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<1.0	<0.1
Ag	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

*The content was determined by TJP-AES method; the relative standard deviation of the results from two experiments were within 5–7%.

**The maximal and minimal values for each metal are marked in bold.

***Mean ± S.D.

$^{\Omega_1}$Sign ≤ in all cases means that the presence of metal in the samples is reliable, but its exact concentration cannot be determined; it can be in the range 0.1–1 μg/g.

Table 7. The relative content of different trace elements and metals in the lyophilized blood plasmas from nine rats.

Metal	Relative content (μg/g)	
	sle-IgG$_{mix}$	**ms-IgG$_{mix}$**
Al	7.0	8.0
Ca	10	120
Cu	8.0	4.0
Fe	4.0	9.0
Mg	4.0	17.0
Mn	0.2	~0
Ni	6.5	0.7
Ti	2.0	27.0
Zn	37.0	11.0

*Preparations sle-IgG$_{mix}$ and ms-IgG$_{mix}$ are mixtures of equal amounts of electrophoretically homogeneous IgGs from the sera of 12 SLE (sle-IgG$_{mix}$) and 12 MS (ms-IgG$_{mix}$) patients.

**The content was determined by TJP-AES method; the errors of the values from two experiments were within 5–7%.

Table 8. The relative content of metal ions in the lyophilized sle-IgG$_{mix}$ and MS-IgG$_{mix}$ samples from the sera of patients with SLE and MS, respectively*.

Nine plasmas of healthy Wistar rat's sera were used for purification of electrophoretically and immunologically homogeneous IgGs according to [31–35]. Homogeneous IgGs according to data of the TJP-AES method did not contain a detectable amount of Sr and Mo (**Table 8**) [31]. The relative amount of different metals bound to IgGs of SLE and MS patients in average decreased in the following order: Ca ≥ Zn ≥ Ti ≥ Mg ≥ Al ≥ Fe ≥ Cu ≥ Ni > Mn (**Table 8**). Thus, IgGs of individual rats can interact with metal ions showing a significant difference (**Table 8**) in spite of their comparable concentrations in the plasmas (**Table 7**).

We have shown that rat IgGs lose most bound metal ions during the purification [31, 33]. From 26 to 39% of rat IgGs can interact with less or more efficiently with different metals ions. Interestingly, chromatography of IgGs from human plasmas on Chelex non-charged with metal ions led to the binding of a small amount of IgGs (~ 5%) bound with metal ions [36]. Chelex charged with Cu^{2+} ions additionally adsorbed ~ 38% of the total IgGs. In a number of publications, it was shown that all many catalytic activities are the intrinsic properties of mammalian antibodies and are not caused by impurities of any canonical enzymes [37–46]. For this purpose, in all cases, we have checked several previously developed strict criteria proving that all activities of Abs from blood sera and healthy donors and autoimmune patients belong to the Abs [37–46].

All higher organisms generate energy due to aerobic respiration, the process including a four-electron stepwise reduction to water of molecular oxygen [47–51]. The partially reduced species include $OH^{\bullet}$, H_2O_2 and $O_2^{\bullet-}$, are typical oxidants attacking proteins, lipids, DNA and other components of different cells. Oxidative damage of cells components was regarded as the significant factor of carcinogenesis and aging [47, 49, 51].

Antioxidant enzymes (catalases, superoxide dismutases and glutathione peroxidases) are very important for preventing oxidative stress [52–56]. However, these enzymes are located inside of cells, and they undergo rapid inactivation in the blood [54]. Immunoglobulins (Igs) are significantly more stable molecules of blood. Therefore, it was interesting how metal ions can activate oxidation-reduction reactions catalyzed by antibodies. The catalysis of such reactions by the majority of canonical enzymes is dependent on metal ions with variable valence [50, 52–55]. First, we have shown that IgGs of healthy Wistar rats oxidize 3,3′-diaminobenzidine through a peroxidase activity in the presence of H_2O_2 and due to an oxidoreductase activity in the absence of H_2O_2 [31–35]. In the external metal ions absence, the specific peroxidase activity of IgGs of rats varied in the range 1.6–26% comparing with horseradish peroxidase (HRP, taken for 100%). The dialysis of IgGs against EDTA completely lost these activities. External metal ions activated significantly both activities of non-dialyzed (ND) and dialyzed (D) IgGs. The relative activities (RAs) in the presence of external Fe^{2+} or Cu^{2+} ions were increased up to 13–198% compared with that for HRP [31]. Cu^{2+} ions alone stimulated significantly both the oxidoreductase and peroxidase activities of dialyzed D-IgGs, but only at high concentration (≥2 mM) [31]. Mn^{2+} ions were weakly activated peroxidase activity but at >3 mM Mn^{2+} was good cofactor of the oxidoreductase activity at a low concentration (<1 mM). Fe^{2+}-dependent peroxidase activity of D-IgGs was revealed at 0.1–5 mM, but Fe^{2+} cannot activate their oxidoreductase activity. Al^{3+}, Mg^{2+}, Zn^{2+}, Ca^{2+}, and especially Ni^{2+} and Co^{2+} were not able to activate D-IgGs, but slightly activated ND-IgGs containing different intrinsic metal ions. Some metal ions activated IgGs especially ND-Abs in accordance with biphasic curves, which were specific for every individual Ab preparation [31]. The combinations of Fe^{2+} + Zn^{2+}, Fe^{2+} + Mn^{2+}, Cu^{2+} + Mn^{2+} and Cu^{2+} + Zn^{2+} and other metal ions led to the oxidation of substrates mainly with single-phase curves. In parallel to a significant increase of the activities comparing with Fe^{2+}, Cu^{2+} or Mn^{2+} taken separately, the RAs of the oxidation reactions catalyzing by non-dialyzed and dialyzed IgGs, became to be comparable. Ni^{2+}, Mg^{2+} and Co^{2+} sufficiently activated the Cu^{2+}-dependent oxidation of substrates catalyzed by D-IgGs, while Ca^{2+} inhibited these reactions [31].

As mentioned above, the dependencies of the oxidoreductase and peroxidase activities of the ND-IgGs and D-IgGs on the concentrations of Fe^{2+}, Cu^{2+} and Mn^{2+} were biphasic. This indicates that two different metal ions are likely to participate in the catalysis of these reactions. The canonical Cu, Zn superoxide dismutases usually use a Cu^{2+} ion together with a Zn^{2+} ion [53–55]. However, the only Cu^{2+} with variable valency participates in the oxidation of substrates, while Zn^{2+} serves as a second electrophilic metal cofactor of this enzyme [53–55]. The biphasic dependences can show for a similar function of the same second or another second metal ion. Since D-IgGs and ND-IgGs demonstrate a significantly higher activity in the presence of Cu^{2+} ions together with Mn^{2+} or Zn^{2+} ions, some fractions of IgGs can be Cu/Cu, Cu/Mn or Cu/Zn peroxidases or oxidoreductases. A remarkable increase in the IgGs activity by Cu^{2+} ions together with Co^{2+}, Mg^{2+} or Ni^{2+} can speak in favor that these metal ions can also increase the oxidative function of Cu^{2+} to some extent as the second ions [31]. Only Fe^{2+} taken separately was activated the peroxidase activity of D-IgGs at low concentrations (<1 mM). However, $FeCl_2$ was completely unable to activate the oxidoreductase activity of D-IgGs. Most probably, Cu^{2+} + Mn^{2+} is an optimal pair for both the peroxidase and oxidoreductase reactions. It seems more likely that the activation of

IgGs by two metal ions with variable oxidation state proceeds either using the second metal as an electrophilic cofactor.

It was demonstrated that small fractions of IgGs from the sera of healthy humans as well as their Fab and $F(ab)_2$ fragments oxidize DAB through peroxidase and oxidoreductase activities [36]. In contrast to rat antibodies, IgGs from human blood have both the dependent and independent on metal ions activities. After dialysis of human IgGs against EDTA and EGTA, the relative peroxidase and oxidoreductase activity dependently of IgG preparation decreased from 100 to ~10–85 and 14–83%, respectively. Addition of external metal ions to D-IgGs and ND-IgGs results in a significant increase in their activities. Separation of IgGs on Chelex results in Abs separation to many different subfractions with different affinities to the chelating resin. In the presence of Cu^{2+} external ions, the specific peroxidase RA of several human IgG subfractions after chromatography achieves 20–27% comparing with horseradish peroxidase (HRP, taken for 100%). The oxidoreductase activity of many IgG subfractions is ~ 4–6-fold higher than that for HRP [36].

It was shown, that IgGs of rats and humans effectively oxidize not only DAB but also many other toxic, carcinogenic and mutagenic compounds such as phenol, *o*-phenylenediamine, α-naphthol, p-hydroquinone, etc. [34]. However, overall, the relative peroxidase and oxidoreductase activities of polyclonal rat IgGs in the presence of different metal ions is ~ 10–100-fold higher than those of polyclonal human IgGs. Interestingly, rats are known as the most resistant mammals to all harmful factors of an environment including carcinogens, mutagens and radiation. One cannot exclude that this is due to better protection of rats compared to peoples from harmful factors due to more active metal-dependent Abs with peroxidase and oxidoreductase activities.

6. Dependence of DNA-hydrolyzing abzymes on metal ions

It was shown in many articles that electrophoretically and immunologically homogeneous polyclonal IgGs from sera of healthy volunteers and experimental mice are not active in the hydrolysis of DNA and RNA (for review see [37–46]). The occurrence of auto-Abs with catalytic activities is a distinctive feature of mammalian autoimmune diseases (reviewed in [37–46]). IgGs and/or IgMs abzymes hydrolyzing DNA and RNA were revealed in the sera of patients with several autoimmune and viral pathologies: SLE [57–61], multiple sclerosis [62–64], Hashimoto's thyroiditis and polyarthritis [65, 66], schizophrenia [67] and with three viral diseases viral hepatitis [68], acquired immunodeficiency syndrome [69] and tick-borne encephalitis [70]), as well as human milk [71–73], SLE mice [74, 75] and experimental autoimmune encephalomyelitis(EAE) mice [76, 77]. Antibodies with DNase activity from the blood of patients and mice with various diseases were dependent on different metal ions [57–70, 74–77], while human milk contains metal-dependent and independent DNase sIgAs and IgGs [71–73]. The RAs of IgGs from the sera of patients (and mice) with different AIDs vary significantly from patient to patient [57–70, 74–77]. **Figure 6** shows the cleavage of plasmid supercoiled (sc)DNA by 10 various IgGs bound with internal metal ions from the sera of patients with various autoimmune diseases.

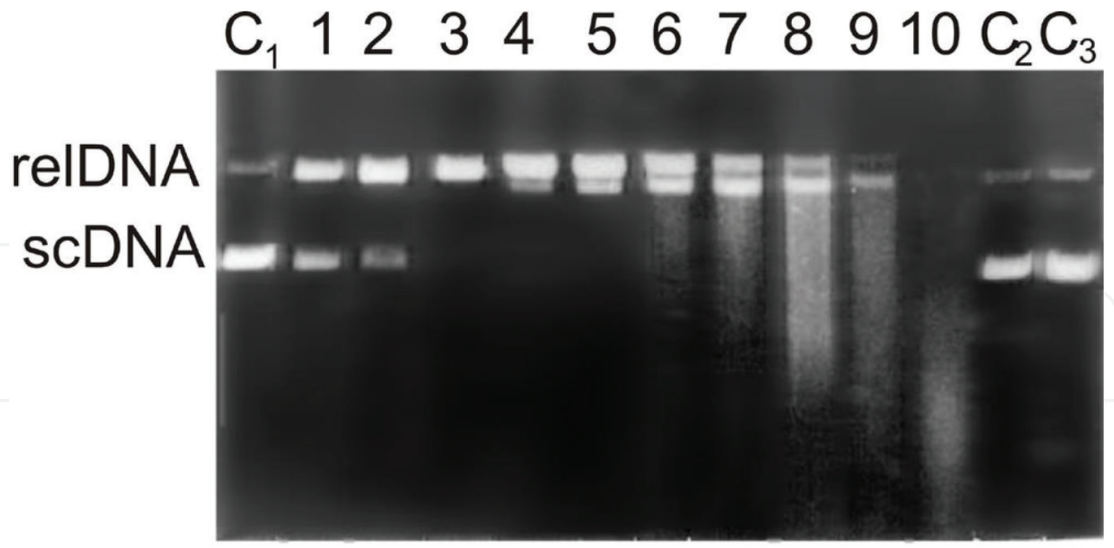

Figure 6. Relative DNase activities of catalytic IgG-abzymes from sera of 10 different patients with various diseases in the hydrolysis of scDNA. Lanes 1–10 correspond to IgGs of 10 different patients; C_1, DNA incubated alone; C_2 and C_3, DNA incubated with Abs of two healthy donors.

During this time, some IgGs cause only single breaks in one strand of scDNA converting it to the relaxed form (lanes 1–3), when others make multiple breaks forming DNA linearization (lanes 4–6). The most active IgGs hydrolyze scDNA into medium- and short-length oligonucleotides (lanes 7–10). Polyclonal DNase IgGs from the sera of autoimmune-prone MRL mice were not after Abs dialysis against EDTA, but were activated by different externally added metal (Me^{2+}) ions: $Mn^{2+} \geq Mg^{2+} > Ca^{2+} \geq Cu^{2+} > Co^{2+} \geq Ni^{2+} \geq Zn^{2+}$ [74, 75]. Fe^{2+} ions could not stimulate the hydrolysis of scDNA by the Abs. The initial rate dependencies on the concentration of different Me^{2+} ions were mostly bell-shaped, having from one to four maxima at different concentrations of Me^{2+} ions. Mn^{2+}, Ni^{2+} and Co^{2+} activated DNA hydrolysis. The Mn^{2+}-dependent scDNA hydrolysis was activated by Ni^{2+}, Ca^{2+}, Co^{2+} and Mg^{2+}, but was inhibited by Zn^{2+} and Cu^{2+}. Only in the case of $Mg^{2+}$and Mg^{2+} or Ca^{2+} as the second metal ions, an accumulation of linear DNA was observed. Affinity chromatography on DNA-cellulose separated DNase mouse IgGs to many subfractions having different affinities for DNA and varying levels of the relative activity (0–100%) in the presence of Mn^{2+}, Ca^{2+} and Mg^{2+} ions. In contrast to all human DNases having 1 pH optimum, mouse IgGs hydrolyzing DNA showed several pronounced pH optima from 4.5 and 9.5; in the presence of Ca^{2+}, Mn^{2+} and Mg^{2+} ions, these dependencies were different. These findings show the extreme diversity of the ability of metal-dependent mouse IgGs functioning at different pHs and to be activated by various optimal metal cofactors. At the same time, a similar situation on an extreme diversity of Me^{2+}-dependent Abs was observed for DNase abzymes from sera of the patient with different autoimmune and viral diseases [70] including monoclonal light chains of human IgGs [78] (e.g., **Figure 7**).

Dependently on patient demonstrated different substrate specificity [63]. All the data obtained showed that polyclonal MS IgGs could contain different combinations of sequence-independent and sequence-dependent endo- and exonuclease activities [63]. The enzymatic properties of the DNA- and RNA-hydrolyzing IgGs of patients with various AIDs [37–46] distinguished them from all known canonical DNases and RNases [79–81].

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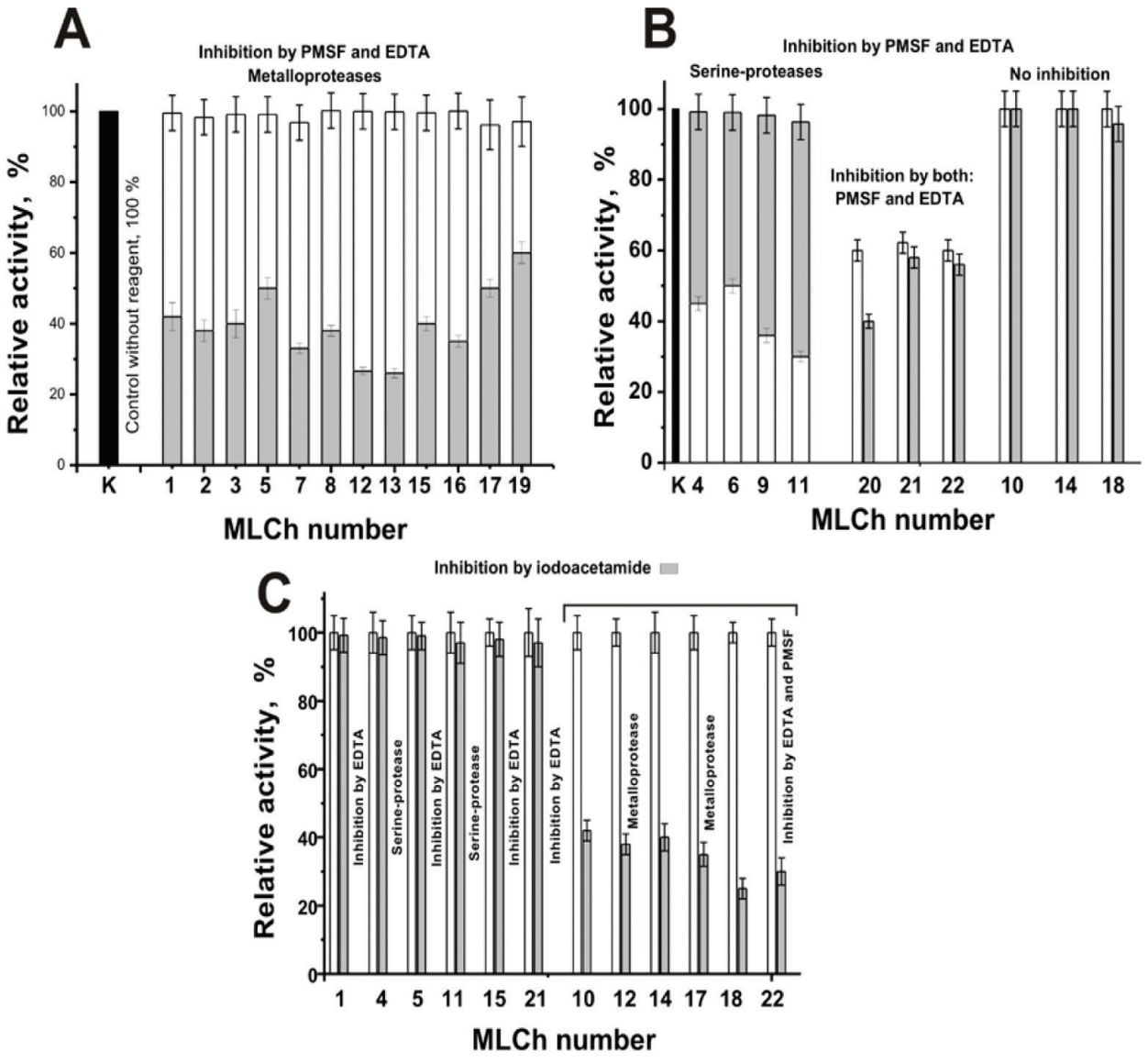

Figure 7. The RAs of MBP-hydrolyzing activity of twenty-two MLChs after their treatment with specific inhibitors of various type proteases. Different MLChs were preincubated in the absence of inhibitors (black bars, control-C), with 50 mM EDTA (gray bars) or 1.0 mM PMSF (white bars) before addition to the standard reaction mixture (A and B). Panel C demonstrates several examples of the RAs of MLChs with metal-dependent (1, 5, 12, 15, and 21) and serine-like activity (4 and 11), which no changing their activity after treatment with iodoacetamide; three MLChs (10, 14, and 18) showing negative response to EDTA and PMSF as well as MLCh-22 having positive answer to PMSF and EDTA after their preincubation with iodoacetamide resulting a significant decrease in the protease activity. Gray and white bars (panel C) correspond respectively to the activity after and before (control) these preparation treatment with iodoacetamide. The Ras of all MLChs before their treatment with different specific inhibitors was taken for 100 %.

Polyclonal DNase IgGs from sera of autoimmune patients, SLE mice, rabbits immunized with DNA and human milk are usually very heterogeneous in their affinity for DNA and can be separated into many subfractions by chromatography on DNA-cellulose [78, 82, 83]. An immunoglobulin light chain phagemid library was prepared using peripheral blood lymphocytes of patients with SLE [78, 82, 83]. Phage particles displaying light chains interacting with DNA were isolated by chromatography on DNA-cellulose; the fraction eluted by 0.5 M NaCl and acidic buffer (pH 2.6) were used for obtaining of individual monoclonal light chains (MLChs, 27–28 kDa) [78, 82, 83].

About 45 of 451 and 33 of 687 individual colonies corresponding to peaks eluted with 0.5 M NaCl and acidic buffer, respectively, were randomly chosen for a study of MLChs with DNase activity. About 15 of 45 (K_m = 260–320 nM) and 19 of 33 (K_m = 3–9 nM) MLChs in the first and second case efficiently hydrolyzed DNA. All 34 MLChs demonstrated different optimal concentrations of KCl or NaCl and pH optima. All MLChs were metal-dependent DNases. The ratio of relative DNA-hydrolyzing activity in the presence of different metal ions was individual for each MLCh. For example, for monoclonal kappa light chain NGK-1 in optimal conditions the RAs decreased in the following order (%): Mn^{2+} (26.3) ≥ Ca^{2+} (23.0) ≥ Mg^{2+} (21.0) > Ni^{2+} (15.0) > Zn^{2+} (11.4) > Cu^{2+} (2.9) > Co^{2+} (0.0) [83]. But in average, the activity in DNA hydrolysis for all MLChs decreased in the following order: $Mn^{2+} > Co^{2+} > Mg^{2+} > Ni^{2+} \approx Ca^{2+} > Cu^{2+} > Zn^{2+}$ [78, 82, 83].

It is known, that Co^{2+}, Mn^{2+}, Ca^{2+} and Ni^{2+} activate mammalian DNase I in much lesser degree than Mg^{2+} ions [80, 81]. Interestingly, human milk polyclonal sIgA DNase abzymes mainly Me^{2+}-independent and they were only slightly activated by Mg^{2+}, Mn^{2+} or Zn^{2+}, and the cleavage of DNA substrates was inhibited by Ca^{2+} and Cu^{2+} [73]. The effect of metal ions on DNase activity of intact Abs from sera of MS patients decreased in the order: $Mn^{2+} > Mg^{2+} > Zn^{2+} > Ca^{2+}$ [84]. The DNA-hydrolyzing activity of tick-borne encephalitis IgGs decreased in the following order: $Mn^{2+} \geq Co^{2+} \geq Mg^{2+} > Ca^{2+}$, while Zn^{2+}, Ni^{2+} and Cu^{2+} did not stimulate DNA hydrolysis [70]. Polyclonal intact IgGs from MRL mice following specific order of DNase activity activation by different metal ions: $Mn^{2+} \geq Mg^{2+} > Ca^{2+} \geq Cu^{2+} > Co^{2+} \geq Ni^{2+} \geq Zn^{2+}$ [74]. Thus, the relative activity of metal-dependent abzymes hydrolyzing DNA depends on the type and timing of the disease as well as can be specific for every individual patient (or animal). On overall, relative metal-dependent DNase activity in blood of patients with different autoimmune and viral diseases increases in the following order: Diabetes < Viral hepatitis ≈ Tick-borne encephalitis < Polyarthritis ≤ Hashimoto's thyroiditis < Schizophrenia < AIDS ≤ Multiple sclerosis < SLE [85].

7. Dependence of RNA-hydrolyzing abzymes on metal ions

First, it was shown that mouse SLE monoclonal IgGs directed against different DNA could effectively hydrolyze both DNA and RNA and cleavage of RNAs is 30–100-fold faster than DNA [86]. Later we have shown that immunization of rabbits with DNA, RNA, RNase A, DNase I or DNase II leads to the formation of abzymes that hydrolyze both DNA and RNA [85]. Interestingly, the substrate specificities of RNase IgGs from patients with autoimmune thyroiditis and polyarthritis [65], SLE [58], MS [62] and hepatitis [68] for different classic homopolynucleotides, cCMP and $tRNA^{Phe}$ with a stable compact structure [39, 59] were different and correlated with the type of disease and were well distinguishable from those of canonical RNases. The activity was strongly dependent on the patient and its disease, but in average increased in the order: hepatitis < polyarthritis < autoimmune thyroiditis < SLE ≤ MS. Abzymes of patients of SLE and MS patients demonstrate new RNase activity stimulated by Mg^{2+} ions [39, 59, 65, 87]. In the presence of Mg^{2+} ions, the abzymes produced products corresponding to new cleavage sites of mutant $tRNA^{Lys}$, indicating its local structural or conformational changes compared to $tRNA^{Lys}$ from mitochondria. Thus, different metal ions play a very important role in the functioning of abzymes with DNase and RNase activities.

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8. Dependence of protein-hydrolyzing abzymes on metal ions

For the first time, elevated levels of polyclonal antibodies to myelin basic protein (MBP) and abzymes hydrolyzing MBP were detected in the blood of MS [88–91] and then of SLE [92–95] patients. In the blood of healthy donors, no such abzymes have been detected [88–95]. It is believed that the mechanism of the pathogenesis of MS is associated with the destruction of myelin (including MBP), leading to inflammation processes associated with autoimmune reactions [96]. Some immunological and biochemical indicators of patients with MS and SLE are very similar [45]. First, we have shown that polyclonal IgG_{mix} (a mixture of equimolar IgGs from 10 MS patients) can hydrolyze MBP in the presence better, than in the absence of different metal ions [88–91]. According to TJP-AES data, homogeneous IgG preparations of MS patients contained several intrinsic metal ions; Fe ≥ Ca > C u ≥ Zn ≥ Mg ≥ Mn ≥ Pb ≥ Co ≥ Ni [90]. Then, a minor Me^{2+}-dependent fraction was obtained by chromatography of one IgG preparation on Chelex-100. This IgG fraction could not hydrolyze MBP in the absence of metal ions but was activated after addition of external $Mg^{2+} > Mn^{2+} > Cu^{2+} > Ca^{2+}$ [90]. Proteolytic activities of individual IgGs from other MS patients were also activated by Fe^{2+}, Ni^{2+}, Zn^{2+}, Co^{2+} and Pb^{2+}, and especially Ni^{2+}. Interestingly, specific proteolytic metal-dependent and independent activities of IgMs and IgAs from sera of MS patient were usually higher than those of IgGs [89]. A significant diversity of different fractions of polyclonal MS IgGs in their affinity for MBP and the hydrolysis of MBP at different optimal pHs (3–10.5) was demonstrating [91]. IgGs containing kappa- and lambda-light chains showed comparable RAs in the hydrolysis of MBP. IgGs of all four sub-classes were active, with their different average contribution to the total activity of abzymes in the hydrolysis of MBP: IgG1 (1.5–2.1%) < IgG2 (4.9–12.8%) < IgG3 (14.7–25.0%) < IgG4 (71–78%) [91]. The properties of MS abzymes demonstrating their significant catalytic diversity distinguish them from all known mammalian proteases including metal-dependent ones. These abzymes can attack MBP of the myelin-proteolipid shell of axons and play an important role in MS pathogenesis [88–91].

At the initial stage of SLE development antibodies against DNA, as well as DNA- and RNA-hydrolyzing antibodies are mostly developed [37–46]. A little later, however, similar to MS pathology in the case of SLE patients the production of Abs against MBP and abzymes hydrolyzing this protein is happening [92–95]. The relative content of different metal ions in the preparations of lyophilized sle-IgG_{mix} and ms-IgG_{mix} from sera of patients with MS and SLE estimated by TJP-AES method to some extent comparable (**Table 8**). Ca^{2+} was the best activator of SLE-IgG_{mix} and its activity increased in the order: $Ca^{2+} > Mg^{2+} \geq Co^{2+} \geq Fe^{2+} \geq Ni^{2+} \geq Cu^{2+} \geq Mn^{2+}$. Zn^{2+} inhibits the activity, while Fe^{2+} cannot activate sle-IgG_{mix}. Ms-IgG_{mix} before dialysis against EDTA showed another order of the activity: $Mg^{2+} > Mn^{2+} \geq Cu^{2+} \geq Ni^{2+} \geq Co^{2+} \geq Ca^{2+}$, while Fe^{2+} and Zn^{2+} slightly inhibit its activity. Thus, on average, patients with MS and SLE develop abzymes hydrolyzing MBP with different dependence on various metal ions. Combinations of Ca^{2+} + Co^{2} and Ca^{2+} + Mg^{2+} results in a significant increase in the MBP-hydrolyzing activity comparing to Ca^{2+}, Mg^{2+} and Co^{2+} or ions taken separately [92]. Lambda-IgGs demonstrated higher RAs in the hydrolysis of MBP than kappa-IgGs [93]. The pH profiles of IgG4, IgG3, IgG2, IgG1 of SLE patients were unique; their RAs increased in the order: IgG4 < IgG2 < IgG3 < IgG1. Thus, the immune systems of SLE similarly to MS patients produce a variety of metal-dependent anti-MBP abzymes, which can hydrolyze MBP of the myelin-proteolipid shell of axons and can play important role in the pathogenesis of MS and SLE patients [45, 46].

Phagemid library derived from lymphocytes of peripheral blood of patients with SLE was used for obtaining of MLChs hydrolyzing MBP [97–100]. About 22 of 72 MLChs hydrolyzing only MBP (not other control proteins) having various pH optima in a 5.7–9.0 range and different specificity in the hydrolysis of four various MBP oligopeptides [97]. Eleven MLChs were metalloproteases, while four and three MLChs showed serine-like and thiol-like proteolytic activities, respectively. The activity of three MLChs was suppressed by both PMSF and EDTA, while the other two by EDTA and iodoacetamide and one by EDTA, PMSF and iodoacetamide. The ratio of RAs in the presence of Mg^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Ni^{2+} and Co^{2+} was very specific for all metal-dependent MLChs. For the total preparation of MLChs, the activity decreased in the order: $Ca^{2+} \geq Co^{2+} \approx Mg^{2++} \geq Mn^{2+} \geq Ni^{2} \approx Cu^{2+} \approx Zn^{2+}$ [97].

In addition to these 22, were isolated other 3 MLChs, which were analyzed in more detailed. NGTA1-Me-pro (MLCh-23) was a typical metalloprotease inhibited only by EDTA [98]. The activity of MLCh-23 in the hydrolysis of MBP was reduced in the presence of ions of seven different metals in the following order: $Ca^{2+} > Mg^{2+} > Ni^{2+} \geq Zn^{2+} \geq Co^{2+} \geq Mn^{2+} > Cu^{2+}$. MLCh-23 has two active sites into the light chain with very distinct pH optima: pH 6.0 and 8.5 and different affinity for MBP [98]. Specific inhibitors of NGTA2-Me-pro-Tr (MLCh-24) were PMSF (42%) and EDTA (58%): it exhibits properties of a chimeric protease with serine and metal-dependent activities [99]. The addition of ions of different metals led to a decrease in the activity of MLCh-24 in the following order: $Ca^{2+} \geq Mn^{2+} \geq Mg^{2+} \approx Co^{2+} \approx Ni^{2+} \geq Cu^{2+} \geq Zn^{2}$. NGTA2-Me-pro-Tr is the first example of an MLCh-23 having two combined centers with serine and metalloprotease activities.

It should be noted that all recombinant MLChs were obtained by affinity chromatography of phage particles on MBP-Sepharose. Taking this into account, a very unexpected result was obtained from analysis of NGTA3-pro-DNase (MLCh-25) [100]. Only 1 MLCh-25 of 25 recombinant MLChs effectively hydrolyzed not only MBP but also DNA. Preincubation of MLCh-25 with both PMSF (67%) and EDTA (36%) resulted in suppression of its protease activity. Ions of different metals activated MLCh-25 in the following order: $Ca^{2+} \geq Ni^{2+} > Co^{2+} \approx Mn^{2+} \geq Cu^{2+} \approx Zn^{2+} \geq Mg^{2+}$ [100]. The affinity of MLCh-25 metal-dependent and serine-like active centers for BMP was different. The DNase activity of MLCh-25 decreases in the following order: $Mn^{2+} \approx Co^{2+} \geq Mn^{2+} > Cu^{2+} \approx Ni^{2+} \geq Ca^{2+} > Zn^{2+}$, which completely distinguishes MLCh-25 from canonical DNases [72]. Metal-dependent casein hydrolyzing sIgA antibodies from human milk were described [101]. The RA of sIgAs after removal of intrinsic metal ions increase their activity in the presence of external $Fe^{2+} > Ca^{2+} > Co^{2+} \geq Ni^{2+}$ and especially combinations of metals: $Co^{2+}+Ca^{2+} < Mg^{2+} + Ca^{2+} < Ca^{2+} + Zn^{2+} < Fe^{2+} + Zn^{2+} < Fe^{2+} + Co^{2+} < Fe^{2+} + Ca^{2+}$ [101].

9. Catalytic activities of antibodies of HIV-infected patients

Metal-dependent IgGs and/or IgMs from the blood of HIV-infected patients hydrolyzing DNA [69], viral reverse transcriptase [102] and integrase [103–105], and all histones [106] were described. Average activities of anti-IN IgGs in the hydrolysis of IN decreased in the order $Mn^{2+} > Mg^{2+} \approx Cu^{2+} > Co^{2+}$ while for IgMs in another order $Cu^{2+} > Mn^{2+} > Co^{2+} \gg Mg^{2+}$. Our findings show that active centers of anti-IN polyclonal abzymes of AIDS patients can contain

amino acid residues providing thiol, serine, acidic and metal-dependent proteases. But the ratio of these abzymes activities may be individual for every HIV-infected patient.

In addition, IgGs from sera of HIV-infected patients hydrolyze all human histones [106]. The RAs of IgGs in the hydrolysis of histones (H4, H3, H2a, H2b and H1) varied significantly for Abs of different patients. The effects of different external metal ions on the dialyzed polyclonal IgGs in the hydrolysis of five individual histones were very different. For example, maximal activation of one IgG preparation was observed in the hydrolysis of H4 by Zn^{2+} and Ni^{2+}, H3 by Cu^{2+} and Ni^{2+}, H2a by Cu^{2+}, H2b by Co^{2+} and Ni^{2+}, H1 by Cu^{2+} and Mn^{2+}. Such an exceptional diversity in activation by different metals ions was observed for all 32 IgGs [106]. Importantly, mammalian immune system theoretically can produce up to 10^6 variants of Abs against one antigenic determinant and all of these Abs may be different.

10. Conclusion

Using the TJP-AES method, we have estimated the relative contents of various trace elements, including metals in various organs, tissues and biological fluids of humans and animals, as well as in immunoglobulins from these sources., the maximal RAs of abzymes with different catalytic activities are most often achieved not in the presence of metal ions, which are contained in biological sources and antibodies in maximum quantities. Some specific abzymes show maximum activity in the presence of metal ions, which are minor elements of different organs and biological fluids. The question is why there are so many abzymes with very different properties including metal-dependent ones against the same protein. First, mammalian immune system theoretically can produce up to 10^6 variants of Abs against one antigenic determinant and all of these Abs may be different. In addition, proteins and nucleic acids can adsorb ions of various metals including traces elements on their surfaces. Therefore, some specific antibodies (and abzymes) can be against fragments (antigenic determinants) of DNA and proteins containing no metal ions. Some other specific metal-dependent abzymes with nuclease and protease activities can be antibodies against sequences associated with one or more metal ions. In addition, not only antibodies against substrates imitating transition states of chemical reactions can possess catalytic activities, but also anti-idiotypic Abs against active centers of various enzymes. The activity of many various enzymes depends on the ions of different metals. Since secondary—anti-idiotypic antibodies against such active sites should contain all the structural components of an enzyme active center including amino acid residues for binding metal ions, they can be metal-dependent abzymes. In this chapter, we have analyzed not only the relative content of different metal ions in various biological substances but also analyzed a possible function of metal ions in the catalysis by autoantibodies of different chemical reactions.

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