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Extracellular presence of aminoacyl–tRNA synthetases

Pozakomórkowa obecność syntetaz aminoacylo–tRNA

Aminoacyl–tRNA synthetases (ARS) /EC.6.1.1.-/ take part in the process of aminoacylation of tRNA. This process takes place in two stages: 1. AA + ATP + ARS ® (AA–AMP:ARS) + PPi, 2. (AA–AMP:ARS) + tRNA ® AA–tRNA + AMP + ARS.

The process of aminoacylation of tRNA by ARS is highly specific. For each amino acid there is a specific aminoacyl–tRNA synthetase which performs aminoacylation of its respective tRNA. Taking into account the number of amino acids which build proteins and the diversity of isoacceptor tRNAs one can understand the role of ARS (1–6). These enzymes also have other functions in the cell. They catalyse the synthesis of diadenosintetraphosphorane (7–8), and some of them autoregulate as well as take part in the maturation of tRNA [9]. Until this time there have been no reports of their extracellular activity or presence. There has not yet been a clear explanation of this fact although anti–ARS antibodies have been found in the blood serum of patients with certain diseases. It can thus be assumed that these enzymes appear in blood serum in a form which does not permit the observation of catalytic activity. The purpose of these studies was to discover ARS activity in blood serum in healthy persons and in those with organ damage as well as to conduct immunological tests in order to observe the presence of these enzymes in blood serum.

MATERIAL AND METHODS

The experiments were carried out on the blood serum of healthy persons and the blood serum of persons with organ damage. Enzymatic preparations of ARS were obtained from human tissues (thyroid gland, gastric mucosa, gastric carcinoma tissue, spleen, colonic carcinoma) which were removed in surgical operations. The comparative tests were performed on ARS which were prepared from rabbit liver. The physiological and pathological blood sera were used to: 1. uncover ARS activity and ARS presence, 2. fractionate blood serum and obtain ARS preparates. The crude ARS preparations were obtained from human tissues. They were purified and fractionated on chromatography columns and high weight complex (HWC) and non–complexed ARS were obtained. ARS samples (HWC and non–complexed) were used to immunise rabbits. The serum thereby obtained contained human anti–ARS antibodies. These serum and immunoglobulin preparates obtained from serum were used to uncover the presence of ARS in human serum.

The tRNA necessary in aminoacylation was extracted from rabbit liver. The control ARS preparates were also obtained from rabbit liver.

Obtaining ARS

The preparation of aminoacyl–tRNA synthetases was obtained by the Borkowski and Charęziński method [10]. The crude ARS preparates were separated by chromatography using Sephadex G–200 or Bio–Gel 1,5 M, and high weight complex and non–complexed ARS were obtained.

tRNA preparation

Preparations of tRNA obtained by phenol extraction from the rabbit liver by the method of Sein and Zubay were fractionated by chromatography on DEAE–52 column (11–12). The concentration of tRNA was determined spectrophotometrically. Additionally, the tRNA was deaminoacylated by the method of Denney (13).

ARS activity

The acceptor activity was tested by using marked amino acids of Dupont Company from Boston, USA. The incorporating system consisted of the following components in total of 200 ml: 100 mM Tris/HCl buffer, pH 7.5, 10 mM $MgCl_2$, 10 mM ATP, 10 mM KCl, 0.4 mM dithiothreitol, 0.1 mM phenylmethylsulfonylfluoride, 1.0 A_{260} units of tRNA, 50 ml of enzymes, and ^{14}C–amino acids (18.5 kBq). In controls the tRNAs were omitted. The incubation was carried out at the temperature of 37°C for 20 min. Next, the samples of 100 ml were applied onto Whatman 3 MM discs which were rinsed four times in cold trichloroacetic acid and then in Hokin fluid (0.8 ml 10 M. NaOH + 62.4 ml glacial acetic acid + ethanol to 1 liter) and ether, and dried. Radioactivity was measured in a scintillation counter of Beckman Company. Activity was determined by the binding of labelled ^{14}C–amino acids by tRNA. The enzymatic activity was expressed by quantity of impulses per minute (cpm) in a tested sample per microgram of protein.

Immunisation of rabbits with human ARS

Adult rabbits, with a weight of approximately 4 kg were used in immunization. Preparations of ARS were given by infusion to a marginal vein of the rabbits'ear. The protein contained in one dose was 200 mg in a volume of 1 ml (to complete the volume 0.9% NaCl was used) (14). Rabbits iminunisations were carried out for four weeks. The antigen injections were performed for three days of each

week. Ten days after the last antigen application (in the fourth week) blood was collected and antibody level was noted. The antibody titre was tested by the Proom method using diluted antigens from 1:100 to 1:32000. 1 ml of blood serum of immunized rabbit was sublayered by 2 ml of the diluting antigen in 0.9% NaCl. The samples were incubated at 37°C for 20 min. and next, were left at room temperature for 10 min. Only blood serums which, with antigen, gave a distinct precipitation ring in dilution 1:8000 were used in further studies.

Uncovering the presence of human ARS by immunological method

Immunodiffusion by Ochterlony method

Immunodiffusion was carried out by using 1.5% gel of agar. The hot agar was poured out on Petri dishes. After cooling down little openings – 6 mm diameter were cut out. Hot agar was poured over the bottoms of these openings. Immunized serum was applied to the central little reservoir. Preparates of tested antigen were applied to the lateral reservoirs. Plates were placed in a moist space and were left at room temperature. The results were read after three and seven days.

Immunoelectrophoresis

The 1.5% agarose gel was used in immunoelectrophoresis. This gel was poured out on the 4 x 12 cm glass. After cooling down little reservoirs were cut out for the antigen and a channel for antibodies was also cut out. The gel was connected to the electrophoretic buffer by 3MM Whatman paper. Veronal buffer 8.2 pH and 0.1 ion strength was used. The electrophoresis of the antigen was carried out for 2–3 hrs at a voltage of 40 V for the plate. Next, immune serum was applied to a channel and the plates enclosed in the moist space. Immunoelectrophoresis was carried out for 72 hrs in the temperature of 20°C and was extended to 7 days. Next, the plates were dried at the temperature of 40°C for 12 hrs.

Quantitative protein testing

The protein was tested colorimetrically by the Bradford method (15).

RESULTS

SEARCHING FOR ARS ACTIVITY IN BLOOD SERUM

Experiments to uncover ARS activity were conducted in complete blood serum as well as protein fractions of blood serum salted out in the range of 40–70%. The physiological blood serum and pathological blood serum in patients with large organ damage, post cardiac infarct patients and neoplasmatic patiens were tested. Although the experiments were repeated many times, no ARS activity was observed in any of the tested complete blood serum nor in the serum fractions.

IMMUNOLOGICAL DETECTION OF ARS IN BLOOD SERUM

DIVIDING ARS INTO FRACTIONS

ARS preparations were obtained from human tissue and rabbit liver by salting out using ammonium sulphate. Next, complete enzyme preparations were obtained removing the salt by dialysis. Next, ARS preparations were divided into complex ARS and free ARS by chromatography, using Bio–Gel. Two large peaks were observed in the fraction (Fig. 1). The first represented macromollecular ARS complex and the second peak represented free ARS.

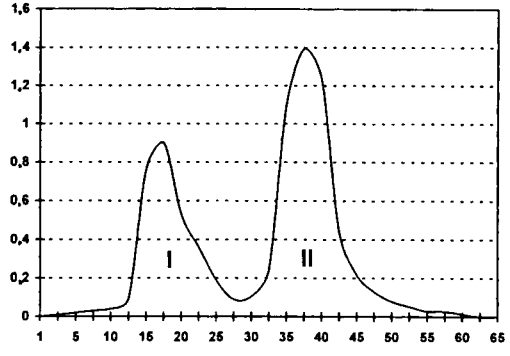

Fig. 1. ARS chromatography on the Bio–Gel 1.5 M. column

Table 1. Titre of rabbits blood serum antibody immunizing with other preparations of ARS

Tissue	Complete preparations of ARS	Fractions of ARS	
		Macromollecular complex ARS	Free ARS
thyroid	1:32000	1:32000	1:16000
gastric mucosa	1:16000	1:16000	1:8000
gastric carcinoma	1:32000	1:16000	1:16000
spleen	1:16000	1:16000	1:8000
colon carcinoma	1:16000	1:16000	1:8000

OBTAINING IMMUNOLOGICAL SERUM

Anti–ARS antibodies were obtained by immunizing rabbits with preparations of ARS obtained from human tissue. Animals immunize well with complete ARS preparations obtained by 40–70% salting out as well as with macromollecular complex ARS and free ARS. The strongest immunization was observed with the complete ARS (Tab. 1).

Specificity of the antibodies was checked by incubating the rabbit serum with ARS preparations used in immunization. After incubation and centrifuge the rabbit serum was checked by immunodiffusion method. There was no precipitation reaction. This demonstrated the specificity of the antibodies since during the incubation period they combined with specific ARS.

UNCOVERING THE PRESENCE OF ARS IN ANTIGEN–ANTIBODY REACTION

The serum and immunoglobulin preparations of immunized rabbits which contained anti–ARS antibodies were used to uncover the presence of ARS in human blood serum. Immunodiffusion in agar gel permitted the obtaining of immunoprecipitates which appeared between anti–ARS antibodies in rabbit serum and tested human blood serum. Since anti–ARS antibodies reacted only with their specific antigen this method demonstrated that ARS is present in human blood serum. The immuno–precipitative reaction is shown in Figure 2.

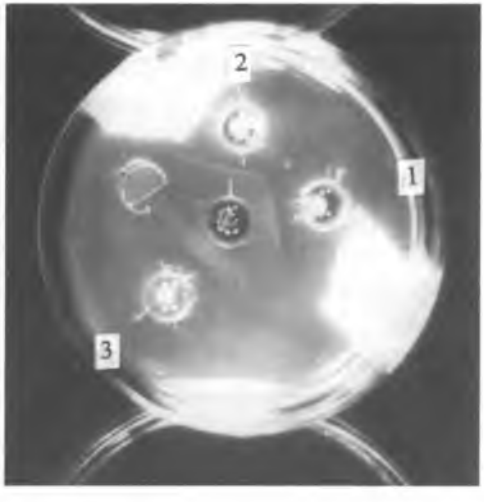

Fig. 2. The immunodiffusion by Ouchterlony method; 1 – control (ARS), 2 – blood serum (cardiac infarct), 3 – blood serum (hyperthyreosis).

The results of immuno–precipitation of the serum of human ARS immunized rabbits with human blood serum are shown in Table 2.

Table 2. Immunodiffusion – intensity of the antigen–antibody reaction

Blood serum	Intensity reaction
Physiological	+
Cardiac infarct	+
Colon carcinoma	+ + +
Gastric carcinoma	+ +
Breast cancer	+ +
Kidney carcinoma	+ +
High aminotransferases	+ +
Hyperthyreosis	+ + +

Arbitrary determination: +++ – very distinct precipitate, ++ – distinct, + – indistinct,

Human blood serum, when tested immunoelectrophoretically, gave a positive result in the form of one precipitate arc. The results of the immuno–precipitation were confirmed by immuno–electrophoresis (Tab. 3).

Table 3. Immunoelectrophoresis — intensity of the antigen–antibody reaction

Blood serum	Intensity reaction
Physiological	+
Cardiac infarct	+
Colon carcinoma	+ +
Gastric carcinoma	+ +
Breast cancer	+
Kidney carcinoma	+
High aminotransferases	+
Hyperthyreosis	+ + +

Arbitrary determination: +++ – very distinct precipitate, ++ – distinct, + – indistinct

These experiments permitted the presence of ARS to be uncovered in blood serum in which ARS was shown to be previously inactive.

DISCUSSION

Blood serum enzymes are present in physiologic and pathologic states. The activity of certain enzymes is greater after gross organ damage (16–17). Until now there have been no reports of determining the activity of extracellular ARS which of course are cytosolic enzymes. Experiments conducted in order to uncover the activity of ARS in physiologic and pathologic blood serum always gave negative results. It is possible that the reason for this is the fact that serum has many components which may affect the aminoacylation environment. In order to eliminate this problem, blood serum was fractionated by salting out with ammonium sulphate in the range of 40–70% (18). No protein fractions, i.e. those salted out in the 40–70% range as well as those salted out in greater and lesser ranges, displayed ARS activity. This may indicate that ARS are not liberated from the cells into the blood circulation. As is well known, ARS display antigenic properties (19–26). Anti–ARS antibodies have been observed in the blood serum of persons with certain autoaggresive diseases such as myositis, polymyositis and interstitial pulmonary inflammation (27–31). On the basis of this fact the decision was made to obtain ARS specific antibodies. Rabbits were therefore immunized using ARS preparations extracted from human tissues such as thyroid, gastric tissue, breast tissue, colon, spleen. The results thus obtained were positive. It was shown that regardless of the tissue of origin, immunologic properties were the same. Keeping in mind that ARS have many forms – complex and free – rabbits were immunized using ARS complex form as well as free ARS. This resulted in the observation that both complex and free ARS cause the occurrence of anti–ARS antibodies. The immunization was more effective when complete preparations and ARS complexes were used and less effective when free ARS were used. The blood serum of immunized rabbits showed high titres in all cases which then permitted them to be used in further tests conducted to determine the presence of ARS in human blood serum. The blood serum of rabbits containing human anti–ARS antibodies as well as the immunoglobulin preparations from these serum samples were used to determine the presence of ARS in physiologic and pathologic human blood serum. Immunoprecipitation in agar gel and immunoelectrophoresis on agarose were the methods used in this experiment. Both methods confirmed that immunoprecipitates between human anti–ARS antibodies in rabbit blood serum and ARS are present in human blood serum. In both cases the intensity of the antigen–antibody reaction depended upon the quantity of antigen and the quantity of antibody. The precipitates were more distinct in samples taken from persons with organ damage. This was because more enzymes, including ARS, were liberated from the cells in pathology state. The search for the presence of ARS in human blood serum gave positive results.

These experiments that proteins with ARS antigenic properties were present in human blood serum.

CONCLUSION

1. Attempts to directly determine activity in the blood serum of healthy persons as well as in those patients who had high activities of other enzymes, gave negative results.

2. Anti–ARS complex antibodies and anti–free ARS antibodies were obtained by immunizing rabbits with preparates of ARS obtained from human tissues.

3. By using the blood serum of immunized rabbits or their immunoglobulin fraction it was determined that human blood serum contains protein with human immunological ARS properties.

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STRESZCZENIE

Syntetazy aminoacylo–tRNA (ARS) ze względu na kluczową rolę, jaką odgrywają w procesie biosyntezy białka, od lat budziły szczególne zainteresowanie. Izolowano je i badano ich właściwości zarówno z materiału roślinnego, bakteryjnego, jak i zwierzęcego. Pomimo zróżnicowania budowy, formy występowania, spełniają one bowiem zawsze swoją zasadniczą funkcję specyficznej aminoacylacji tRNA. ARS w komórce występują głównie w cytosolu, ale znajdują się także w mitochondriach. Dotychczas nie stwierdzono jednak ich pozakomórkowej obecności. Od kilku lat pojawiają się natomiast doniesienia o obecności przeciwciał przeciwko niektórym ARS w surowicy krwi ludzi chorych na pewne schorzenia z autoagresji. Właśnie te powody były przyczyną podjęcia badań, mających na celu: 1) próby wykrycia pozakomórkowej obecności ARS i 2) przeanalizowanie możliwości uwalniania ARS z komórki. Na wstępie przeprowadzono badania aktywności ARS w surowicach krwi ludzkiej fizjologicznych i patologicznych (chorych z dużymi uszkodzeniami narządowymi). Zarówno w surowicach pełnych, jak i ich frakcjach nie stwierdzono aktywności enzymatycznej ARS. Brak aktywności nie wykluczał ich obecności w surowicy krwi. Przeprowadzono więc badania mające na celu wykazanie obecności ARS metodami immunologicznymi. Z niektórych tkanek ludzkich otrzymywanych z klinik chirurgicznych uzyskiwano preparaty ARS, którymi immunizowano króliki. Otrzymywano surowice zawierające przeciwciała, które reagowały zarówno z frakcją wolnych ARS, jak i z frakcją kompleksu wysokomolekularnego. Przy użyciu techniki immunodyfuzji i immunoelektroforezy wykazano w badanych surowicach ludzi zdrowych i w surowicach patologicznych obecność ARS. Pozostaje nadal nie wyjaśniony problem braku aktywności ARS w surowicy krwi.