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TRANSLATION NO. 2217

DATE: October 1967

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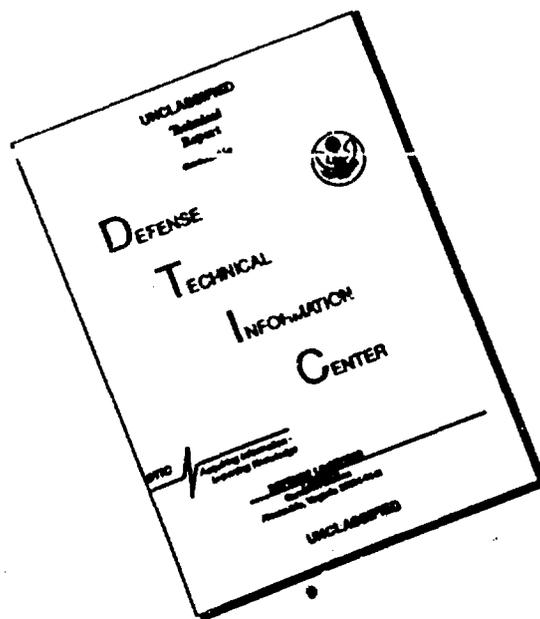
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## ANTIBODIES AGAINST NUCLEIC ACIDS

[Following is the translation of an article by M. I. Levi, Rostov/Don Scientific Research Institute (Antiplague), published in the Russian-language periodical Voprosy Virusologii (Problems of Virology) No 3, 1965, pages 259--265. It was submitted on 28 May 1964. Translation performed by Sp/7 Charles T. Ostertag, Jr.]

The protein components of viruses possess expressed antigenic and immunogenic properties, therefore, the detection of antibodies against them does not cause any difficulties. Nevertheless, up until the present time, it is still unclear what mechanisms the microorganism uses in combatting nucleic acids, which guarantee the intracellular multiplication and preservation of the hereditary properties of viruses. It is possible that the destruction of viral nucleic acids is provided for by nucleases /7/. The latter may turn out to be inactive against the intact virus /17/, in which the nucleic acid is surrounded by a protein membrane. However, it is known that nucleic acid as a necessary intracellular stage in the development of the etiological agent also exists in the organism in an "exposed" condition. It is not ruled out that the organism has special immunological mechanisms, directed at the destruction of viral nucleic acids.

The peculiarities of immunity are noted during lymphocytic choriomeningitis. Thus, in white mice, in contrast to other animals, only complement-fixing, but not virus-neutralizing, antibodies are formed; the soluble antigen of the lymphocytic choriomeningitis virus is specific for it, but non-infectious, and when animals are immunized with it, in their serum there is an accumulation of complement-fixing, but not virus-neutralizing antibodies; if the soluble antigen is added to immune serum, containing both types of antibodies, then only the complement-fixing antibodies are inactivated; in man and animals the complement-fixing antibodies are detected in 3--4 weeks after the onset of the disease, and virus-neutralizing antibodies - in 9--11 weeks. In connection with this, we proposed that ".....there are known differences between complement-fixing and virus-neutralizing antibodies. Moreover, the complement-fixing antibodies apparently correspond to active groups of the protein membrane of the lymphocytic choriomeningitis virus, and the virus-neutralizing antibodies are directed against the viral nucleic acid, with which the biological activity of this type of antibody is connected" /4/. However, this assumption is contradicted by the fact

that while virus-neutralizing antibodies are almost always highly specific, the majority of researchers, using various immunological methods, have not been able to distinguish between nucleic acids, isolated from various biological objects, for example, the DNA of viruses from the DNA of bacteria or mammals /1/.

Since at the present time there has been no success in detecting species differences in nucleic acids by using immunological methods, almost all the investigations in this field have been carried out on models of nucleic acids, isolated from the tissues of laboratory animals or from bacterial etiological agents.

The history of the discovery of antibodies against DNA bears the imprint of chance. Antibodies to various human antigens have been detected in the sera of patients with disseminated lupus erythematosus [6, 16, 19, 27]. In such sera antibodies to cell nuclei, nucleoproteins and DNA were found [16, 19, 33]. Beginning with 1957, an intensive study has been made of the antigenic properties of DNA, however, all attempts to detect the accumulation of antibodies in animals as a result of the artificial introduction of purified DNA or synthetic polyribonucleotides, and also artificial conjugates of protein and DNA, usually ended with failure, possibly due to the "ubiquitousness" of DNase [7, 23--25]. We also were not able to obtain the accumulation of antibodies when rabbits and guinea pigs were parenterally injected with complete Freund's adjuvant and preparations of native or denatured DNA, both by themselves and in a mixture with a neutral solution of sodium citrate. Several investigators were able to verify the presence of antibodies against DNA in those cases when animals were immunized, not with purified preparations of DNA, but with nucleoproteins [28]. In connection with what has been presented above, the majority of investigators consider that DNA is not a complete antigen, but only plays the role of a haptén [23, 26].

It was possible to detect antibodies to DNA by the precipitation reaction in liquids and gels [18, 20] by the complement fixation reaction [9, 10, 18, 31, 38], the conglutination reaction [28, 32], the agglutination reaction of bentonite and latex, sensitized with DNA, and also the passive hemagglutination reaction [11, 14, 15, 23, 26], and by the immunofluorescent method [21, 30]. Recently, American investigators successfully utilized a micro-modification of the complement fixation reaction for these aims [22, 38]. The micro-modification consists of the following. A small amount of erythrocytes is taken for preparation of the hemolytic system. The degree of hemolysis is calculated with the help of a spectrophotometer at a wave length, characteristic for oxyhemoglobin (413 m $\mu$ ), and the amount of added complement corresponds to 50--80% of the hemolytic dose. The first phase of the reaction lasts 18 hours at 4 $^{\circ}$ , then the hemolytic system is added

to the test tubes and they are transferred to a water bath (37°). After 30 minutes the contents of each test tube are centrifuged, placed in cuvettes and extinction measurements are made at a wave length of 413 m [37]. The micro-modification of the complement fixation reaction made it possible to detect antibodies in the diluted serum of patients with systemic lupus erythematosus (1:200 - 1:400) in the presence of a trivial amount of DNA (0.01 g). It is not recommended to use large doses of DNA due to the expressed zonality and possible anticomplementary activity of the antigen. The complement fixation inhibition reaction was set up for studying specificity, and also for investigations directed at establishing determinative groups of DNA.

In recent years the passive hemagglutination reaction has become widely distributed in serological investigations /5/. Lawlis [27] was the first to carry out the sensitization of erythrocytes with the help of DNA. He noticed that DNA is adsorbed only at a low pH. Lawlis worked with human formalinized erythrocytes. Later several investigators [11, 23] could not obtain satisfactory results with the help of this reaction. A. M. Poverenny and M. I. Levi /8/ demonstrated that, after a preliminary treatment with tannin, formalinized sheep erythrocytes adsorb DNA at a low pH, and suchsensitized erythrocytes are agglutinated in the presence of serum from a patient with systemic lupus erythematosus, which contains antibodies to DNA. If such DNA-sensitized erythrocytes were submerged in an 0.4% solution of formaldehyde, the activity of the preparation did not change over a period of several months.

Thanks to the stability of a preparation of sensitized erythrocytes, the setting up of serological reactions, based on its investigation, was immeasurably simpler, and the results of the reaction were characterized by a relative exactness and reproducibility. For studying the antibodies to DNA, A. M. Poverenny and M. I. Levi /8/ used the passive hemagglutination reaction, and for the detection of DNA, the antibody neutralization reaction, which made it possible to expose such small quantities as 0.01--0.1 g of DNA. This corresponds to approximately  $10^7$  molecules of nucleic acid. It was shown that treating sensitized erythrocytes with DNase greatly lowers their activity in the passive hemagglutination reaction, and treatment of DNA with this enzyme makes it inactive in the antibody neutralization reaction. Important proofs of the specificity of serological reactions were obtained in tests on the renaturation of DNA. As is known, if microbial DNA, which was denatured by boiling, is slowly cooled without formalin, the restoration of the double spiral structure of the nucleic acid takes place, while with the DNA of higher animals, this is not observed /2/.

In these tests, if slow cooling was employed, it was possible to note changes in the neutralizing activity of preparations of denatured bacterial DNA in a direction approximating the indices, characteristic for native DNA. Under those conditions, denatured DNA from chicken erythrocytes does not renature. The results of these and other experiments

convinced investigators of the specificity of serological reactions used for studying the antigenic properties of DNA. Stollar, Levine and Marmur and Stollar and Levine [36,38] detected differences in the properties of sera from systemic lupus erythematosus patients. The sera reacted with various quantities of DNA. A. M. Poverenny and M. I. Levi [8] detected two types of antibodies: In the sera of some patients antibodies could be neutralized by small quantities of single spiral DNA and relatively large quantities of native DNA (type I), in the sera of other patients the antibodies could be neutralized by approximately the same quantities of single and double spiral DNA (type II). Antibodies to double spiral DNA were found only in several systemic lupus erythematosus patients. These died in several months. Barbu et al. [12] initially found antibodies to native DNA in some patients and therefore considered that the denaturation of DNA leads to the loss of antigenic qualities, but then they, just as Stollar, Levine and Marmur [36], detected that the sera of SLE patients reacts not only with DNA which has been denatured by boiling, but also with native DNA of phage  $\phi$  174, which has a single-stranded structure.

As is known, DNA is concentrated in the nucleus of the cell. Rapp and Friend [30] used the immunofluorescent method (indirect) for the detection of DNA-containing viruses in the protoplasm of infected cells. For this the sera from SLE patients was poured into the appropriate media, after which the preparations were treated with antihuman horse pseudoglobulin, combined with fluorescein isothiocyanate. This method has specific advantages for the detection of DNA-containing tumefacient viruses.

At the same time that Rapp and Friend [30] and Gikcen [21] found that antibodies to DNA penetrate through cell membranes, Williams and Bollum [38] detected that these antibodies do not hinder the growth of normal or tumoral cells in vitro, however, they hinder the synthesis of DNA outside of the cells in the presence of primer and polymerase.

In connection with the increase in the activity of the erythrocytic diagnostic agent and the application of absorption of the sera under test by sheep erythrocytes, M. I. Levi and A. M. Poverenny detected, in low titers, antibodies to single spiral DNA in the sera of the majority of healthy persons and laboratory animals (see table).

It was demonstrated with these sera that for the neutralization of 1--2 serumal units it required 0.015--0.06 g of single spiral DNA of various origins, while native DNA or preparations of RNA are inactive even in doses of 40 g. Thanks to the discovery of antibodies to single spiral DNA in the blood of healthy persons and animals, a practically inexhaustible reserve of sera was obtained. This reserve is necessary for various purposes, including for the standardization of prepared series of erythrocytes, sensitized with DNA. Previously the sera from SLE patients was always the source of antibodies to DNA.

These same authors found that in guinea pigs, the titer of antibodies to single spiral DNA increases with age. It was shown in several works that antibodies to DNA were contained in the gamma-globulin fraction, withstood heating at 60°, but were destroyed as a result of heating at 70° for 30 minutes. Formaldehyde in concentrations exceeding 0.8% inactivates the antibodies.

It could have been expected that the antibodies to the DNA detected are produced in the lymphoid tissue, however, the water-saline extract from the spleen and lymph nodes of laboratory animals, the sera of which contained antibodies to DNA, were inactive in the passive hemagglutination reaction, inasmuch as these extracts, prepared in a physiological solution with sodium citrate, possessed a neutralizing activity in the antibody neutralization reaction with sera, containing antibodies to single spiral DNA, and with sera, containing antibodies to native DNA. Thus, it was not possible to expose antibodies in extracts of lymphoid organs due to the presence of antigenically active derivatives of DNA in them. Fisher, Fisher and Samuelson [20] detected an antinuclear factor in the lymph of dogs which was running out from a fistula in the thoracic duct. Naturally such a method of detecting antibodies to DNA is more justified than searching for them in tissue extracts.

There is considerable interest in the problem concerning the determinant groups in a molecule of DNA which are responsible for antigenic properties. Deamination with nitrous acid, methylation with dimethyl sulfate /8/ did not have an effect on antigenic activity. Barbu et al. [12] detected that polyphosphate (25 radicals of phosphoric acid) does not possess an antigenic activity in serological reactions. The influence of ultrasound, radiation with ultraviolet rays, treatment with 10% formalin, alkalizing to pH 12.0 and acidification to pH 1.5, based on the data of Barbu et al. [12], did not influence the capability of DNA to enter into serological reactions with the sera from SLE patients. During depurination, a lowering took place in the capability of DNA to react with antibodies. Stollar and Levine [36] found that synthetic polyadenylic acid and apurinic acid, obtained from DNA, do not react with antibodies to DNA. In the suppression of the complement fixation reaction the purines were more effective than the pyrimidines. Grossman, Stollar and Herrington [22] showed that in a molecule of DNA, ultrasound changes pyrimidine to a greater degree than it does purine. This may explain the [circumstance] that DNA, treated with ultrasound, does not change its serological activity. Since RNA does not neutralize antibodies to DNA, Stollar et al. [35] proposed that fragments of the polymer which contain thymine as the nitrogenous base possess antigenic properties. Synthetic tetra- and pentathymidylic acids inhibit the complement fixation reaction with the serum of a SLE patient and DNA.

Bigley, Dodd and Geyer [14] detected that the inhibition of the passive hemagglutination reaction with antibodies against cell nuclei is brought about by thymine monophosphate. Antibodies against RNA may be neutralized by uridine, uridine monophosphate and uridine triphosphate. However, these data require confirmation, since the method of serological reactions used by the authors, and the doses of antigen and antibodies differed from those usually accepted.

We did not observe the neutralization of antibodies to DNA in the presence of various mononucleotides, including thymidylic acid. A. M. Poverenny, Oleynikova and M. I. Levi found that adenine-thymine pairs of nucleotides have a relationship to the determinant groups in a molecule of DNA. Such a conclusion was made on the basis of observations on the prolonged thermal denaturation of DNA samples with a various nucleotide composition at suboptimum temperatures in the presence of formalin. In these tests it was possible to demonstrate that following the separation of the DNA spirals into fragments, rich in adenine-thymine nucleotide pairs, quantitative changes in antigenic activity did not occur though in subsequent periods the separation of DNA spirals continued mainly due to fragments, rich in guanine-cytosine nucleotide pairs. An analysis of mutations in bacteria led to a conclusion concerning the existence of "genetically blind" uncoded fragments of the polynucleotide chain of DNA, which based on the proposal of N. P. Dubinin [3] are made up of repetitions of one pair of nucleotides. It is possible that such uncoded fragments of DNA may have real importance in the determination of antigenic properties for this polymer. A. M. Poverenny and M. I. Levi [8] proposed that large fragments of DNA, sometimes found in various polynucleotide chains, are included in the composition of the determinant groups. On the basis of experiments with DNase, Barbu et al. [12] came to the conclusion that the determinant groups of DNA contain from 2 to 50 nucleotides.

Based on the evidence of a number of authoritative investigators [12, 14, 27, 36], in different SLE patients the sera possess various properties - the antibodies in some of them are suppressed by mononucleotides; with other sera this was not observed. Some authors [36] even note that various sera are bound with various amounts of DNA. Thus, for some the optimum is 0.02 g, for others - 1 g. Moreover, the sera of SLE patients contain antibodies to whole cells, isolated nuclei, DNA, nucleoproteins and histone, and according to some authors each of the above antigens has its own antibodies [19, 31]. Gokcen [21] utilized the immunofluorescent method and the sera of SLE patients for proof that there are various antibodies to DNA; specifically he detected antibodies to the DNA of neutrophils and antibodies to the DNA of human lymphocytes. According to the assumption of the author, the DNA in these leukocytes have different structures. Apparently the subsequent process of identifying the determinant groups of DNA will be connected not only with defining the active groups of a molecule of nucleic acid, but also with the purification and analysis of the antibodies against DNA.

Barbu and Panijel investigated the antigens of ribosomes of various viruses and it turned out that the ribosomes had the same common antigen, which was identified as RNA. In tests with the precipitation reaction in gel, it was demonstrated that the antigenic properties of RNA do not depend on its species affiliation [13, 14, 29]. Based on the data of Barbu, Quash and Dandeuy [13], the antibodies against RNA are specific. The sera of animals, immunized with ribosomes, reacted with synthetic polynucleotides. With the help of precipitation in gel and liquids, Barbu et al. showed that antibodies to RNA are contained not only in the sera of animals, immunized with ribosomes, but also in the sera of healthy persons and animals (rats, rabbits, sheep and horses). In those cases when the precipitation reaction in liquids was used, the quantitative determination of antibodies was performed with the help of the calculation of protein in the precipitate. In experiments on chick embryos and chicks of various age, Barbu et al. showed that the antibodies to RNA are contained in the blood of embryos before hatching of the chicks, and then their titer increases with age (observations were conducted up to the 30th day).

An outstanding fact was the establishment of a heterogeneity of antibodies to RNA, demonstrated by differences in the capability to precipitate certain nucleic acids. This provides a foundation to consider that different species of RNA may be differentiated not only by biochemical, but also by immunological methods. In particular, it can be proposed that the transport RNA and the Ribosomal RNA may be "identified" with the help of specific immune sera to them.

Bigley, Dodd and Geyer [14] confirmed the data of Barbu et al. concerning antibodies to RNA by using the passive hemagglutination reaction with fresh human erythrocytes. The adsorption of nucleic acid to erythrocytes was carried out with the help of twofold diazotized benzidine at neutral pH values. Bigley et al. came to the conclusion that during various autoimmune diseases, there occurs the formation of antibodies to nucleic acids, bound to microsomes, ribosomes and nuclei. In rabbits which had received yeast RNA in Freund's adjuvant, the authors found the symptoms of autoimmune illness against a background of a growth in the titer of antibodies to RNA.

Thus it has been firmly established that nucleic acids (DNA and RNA) have the capability to form specific complexes with the corresponding antibodies, which is documented by various serological reactions. Of the serological reactions used, preference belongs to the passive hemagglutination reaction and the antibody neutralization reaction, based on the utilization of an erythrocytic diagnostic agent. Along with data concerning the impossibility of using immunological methods to differentiate DNA, obtained from various sources, information has accumulated in the literature concerning quantitative differences in nucleic acids (DNA and RNA), which play a various physiological role in the organism. In

connection with this, we have a right to assert that there exists a whole group of antigens - nucleic acids -- united by a similarity in chemical structure, to which there is a corresponding group of antibodies, possessing known varieties. Therefore, it is quite justified to study the antigenic properties of viral nucleic acids. In particular, American investigators have established that the DNA of T-even phages, which contains glucosylated 5-hydroxymethylcytosine, is easily distinguished in immunological tests from other preparations of DNA, containing ribose. [1, 22]. It may be thought that the study of the antigenic activity of nucleic acids from viruses of animal, plant and bacterial origin will render a significant help in decoding the molecular structure of these biopolymers.

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Antibodies to single strand DNA  $\sqrt{S}$  in the sera of healthy persons and laboratory animals

Sera	Number of sera	Distribution of sera according to the activity of the passive hemagglutination reaction based on a titer of						
		0	1:10	1:20	1:40	1:80	1:160	1:320
Man	587	263	77	132	82	26	7	-
Guinea pig	130	27	7	21	35	26	11	3
White rat	65	20	-	-	1	2	15	27
Golden hamster	52	42	4	3	3	-	-	-
Rabbit	33	14	5	12	2	-	-	-
White mouse	4	1	-	1	-	1	-	1