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INDICATION OF VIRUSES AND VIRUS-SPECIFIC ANTIBODIES BY ELISA USING CONJUGATES BASED ON β-LACTAMASE OBTAINED BY GENETIC ENGINEERING

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The method of enzyme-linked immunosorbent assay (ELISA), by means of which antigens and antibodies of different origin can be detected with high sensitivity and specificity in biological samples, has achieved widespread popularity in recent years. Virtually all varieties of immunoenzyme techniques are based on the use of conjugates, which are macromolecular complexes formed by covalent "attachment" of enzyme molecules to antigen or antibody molecules. Enzyme-labeled macromolecules, immobilized during the assay, can be detected by adding chromophore, fluorocheome, or radioactive substrates, modified by the chosen enzyme, to the system [1].

Conjugates based on peroxidase, alkaline phosphatase, and β-galactosidase are most frequently used at the present time to construct immunoenzyme test systems [10]. However, the use of these enzymes (in particular, peroxidase, which is most widely used) in ELISA is complicated by the fact that they are often present in the free or bound form in the biological material to be studied [2], and also by the fact that substrates of these enzymes in most cases either possess low stability, or are difficult to synthesize, or are toxic to the operator [1]. Accordingly, the search for new enzyme-substrate pairs for use in ELISA techniques still remains an urgent task.

Most recently conjugates containing β-lactamase (penicillinase, penicillin-amido-β-lactan hydrolase, EC 3.5.2.6) have begun to be used in the construction of new immunoenzyme test systems [3, 14, 15]. This enzyme, whose amino-acid sequence and principal physicochemical properties are known [8, 13], hydrolyzes the β-lactam ring of penicillin and its derivatives, and converts them into penicillonic acids, which is detectable by a relatively simple iodometric method [6]. The chief advantage of using lactamase conjugates is the simplicity and convenience of the substrates used for its detection — mixtures of penicillin, starch, iodine, and potassium iodide [15] or cadmium iodide [3].

The source of the enzyme used to synthesize lactamase conjugates is usually penicillin-resistant strains of bacteria [3, 14, 15]. In the present investigation we used for this purpose β-lactamase, synthesized by a genetic engineering method, which has many advantages over the traditional methods of obtaining the enzyme. We used the lactamase conjugate in immunoenzyme test systems intended for the detection of viruses and virus-specific antibodies in biological specimens (influenza virus was used as the model). It was also natural to compare the sensitivity of immunoenzyme methods based on β-lactamase conjugates and those

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based on conjugates including horseradish peroxidase, which has traditionally been used in ELISA.

The enzyme used in this investigation to synthesize the lactamase conjugate (β-lactamase of the TEM-1 type) was obtained from *E. coli* cells (strain W3101) as follows [7]: the bLa gene, responsible for β-lactamase synthesis, was inserted into the phage λ genome at EcoRI sites in the composition of plasmid PCV-II. In the process of reproduction of the phage in infected bacterial cells, molecules of β-lactamase were synthesized, and toward the end of fermentation they amounted to 600 mg of enzyme per liter of culture fluid. The enzyme was purified by differential precipitation with ammonium sulfate and chromatography, using DEAE-cellulose as the carrier [13].

The influenza virus used in the work (strain A/Phillippines/2/82), the electrophoretically pure nucleoprotein protein (NP-protein) of influenza virus, and monospecific rabbit serum to NP-protein were obtained by methods described previously [4, 5].

The peroxidase conjugate was obtained by the periodate method [12], using a Soviet preparation of horseradish peroxidase (from the Olaine factory), with R = 3.0, and donkey anti-rabbit IgG (produced by the N. F. Gamaleya Institute of Epidermiology and Microbiology, Academy of Medical Sciences of the USSR). These same antibodies were used to obtain a lactamase conjugate, which was synthesized by the glutaraldehyde method [10], after which both conjugates were purified by a column with Sephadex G-50.

The conditions of ELISA were as follows: adsorption of antigen (rabbit immunoglobulins, influenza virions) — 16 h, 4°C; incubation with anti-HP-immune and control (normal) rabbit sera — 1 h, 37°C; adsorption of the conjugates (dilution 1:1000) — 1 h, 37°C; incubation with the substrates — 4 h, 37°C for the β-lactamase conjugate and 20 min, 37°C for the peroxidase conjugate. A mixture of 0.05% orthophenylenediamine (Sigma, USA) and 0.003% H_2O_2 in 0.06 M phosphate buffer, pH 5.9, was used as the substrate for peroxidase; the substrate for lactamase was a mixture of 0.1% water-soluble starch, 0.16% KI, 0.006% I , and 0.003% benzylpenicillin (all reagents were of Soviet origin) in phosphate buffer, for ELISA [1].

The sensitivity of the ELISA methods was determined as the minimal quantity of antigen in a well for which the optical density exceeds the mean optical density of the control (usually 10 control samples of uniform type were analyzed) by three standard deviations. One aim of this investigation was to compare the sensitivity of a standard peroxidase test system and that of a system in which β-lactamase, immobilized on antispecific antibodies was used as the conjugate. We used these systems to detect, with the aid of the modified double-sandwich [1] method, rabbit immunoglobulins, virus particles (influenca virions were used as the model), and virus-specific antibodies (antibodies to NP-protein influenza virus, contained in rabbit serum). The results of this type of comparison of sensitivity of two immunoenzyme test systems are given in Figs. 1, 2, and 3. On the basis of this experimental material it can be concluded that the lower limits of determination of the different antigens were about the same for the two test systems, and were, for rabbit immunoglobulins, 0.1 ng per sample, and for influenza virions, 1-2 ng as total virus protein (Figs. 1 and 2). These same test systems were able to reveal virus-specific immunoglobulins when used on immune rabbit anti-NP-serum in a dilution of 1:2,560,000 (Fig. 3).

Besides guaranteeing high sensitivity of the immunoenzyme test systems constructed with their use, lactamase conjugates also have many important advantages over conjugates based on horseradish peroxidase, alkaline phosphatase, and β-galactosidase widely used at the present time. These advantages are as follows.

1. The enzyme β-lactamase can be isolated relatively easily from a wide range of strains of bacteria, with a high yield of the isolated product [11]. In addition, as the present investigation has shown, an enzyme synthesized by genetic engineering can be used to synthesize lactamase conjugates. The high productivity of enzyme-synthesizing systems in this case (and, consequently, the relatively low cost of the end product) and the possibility of modifying the synthesized polymer (in particular, synthesis of its molecular fragments) at will provides new opportunities for the construction and industrial production of immunoenzyme kits for use on a wide practical scale.

2. The genetic engineering method of synthesis of β-lactamase, condidering its low molecular weight (28,900 daltons), opens up prospects for the synthesis of immunoenzyme conjugates of a new type, namely hybrid molecules incorporating complete or partial amino-acid sequences of the enzyme and antigen simultaneously. The theoretical basis for the production

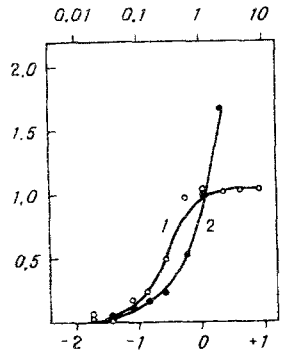

Fig. 1

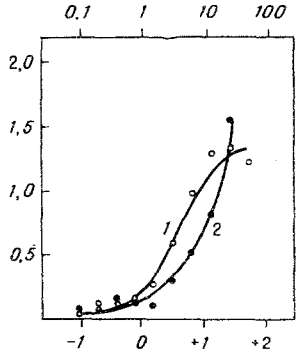

Fig. 2

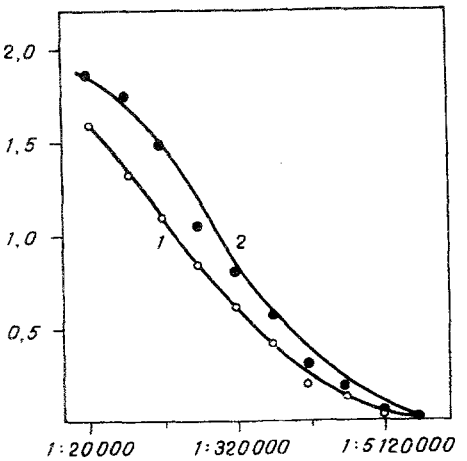

Fig. 3

Fig. 1. Comparison of sensitivity of lactamase (1) and peroxidase (2) test systems during indication of rabbit immunoglobulins. Horizontal axis: above — quantity of immuloglobulins in well (in ng); below — its logarithm; vertical axis (here and in Figs. 2 and 3) — optical density. Into wells of polystyrene plates (Dynatech, USA) were added successively rabbit IgG, BSA, conjugate (dilution 1:1000), and substrate. Because the result of the action of β-lactamase consists of decolorizing an initially bright blue solution, to read the results of the reaction, the difference in optical densities (OD) of the control and experimental samples was used (photometry at 570 nm); for the peroxidase conjugate the results were read by using the value of $OD_{exp}-OD_{cont}$ (photometry at 492 nm). Wells not containing rabbit immunoglobulins were used as the control.

Fig. 2. Comparison of sensitivity of lactamase (1) and peroxidase (2) test systems during indication of influenza virus. Horizontal axis: above — quantity of total virus protein in well (in ng), below — its logarithm. Into wells were successively added influenza virus, BSA, immune anti-NP-rabbit serum (dilution 1:20,000), conjugate (dilution 1:1000), and substrate. In control, instead of immune serum, normal rabbit serum was used in the same dilution. The results were read as indicated in the caption to Fig. 1.

Fig. 3. Comparison of sensitivity of lactamase (1) and peroxidase (2) test systems during indication of virus-specific antibodies in rabbit anti-NP-serum. Horizontal axis — dilution of serum. Into wells were added 50 ng of influenza virus, followed in succession by immune serum, conjugate (dilution 1:1000), and substrate. Control — normal rabbit serum in the same dilution. The results were read as indicated in the caption to Fig. 1.

of conjugates of this type, at least for antigens of influenza virus [9], has already been worked out.

3. β-Lactamase is synthesized only by penicillin-resistant strains of microorganisms and, unlike many other enzymes widely used in ELISA, it is not present in ordinary biological and clinical specimens. This situation is extremely important for the development of diagnostic immunoenzyme test systems (especially for the detection of plant viruses), when the presence of the free or bound enzyme, included in the conjugate, in the test system would lead to "illumination" of both the experimental and the control specimen.

4. The nonlinear relationship between the chromophore response of the lactamase test system and antigen concentration with a narrow interval of lowering of the optical density of the solution (Figs. 1 and 2) suggests that lactamase conjugates will be particularly convenient for the development of immunoenzyme test systems with visual monitoring of the results of the assay, although this does create definite difficulties for quantitiative determination of the antigen in biological and clinical specimens.

5. Disadvantages of immunoenzyme test systems using lactamase conjugates include the longer incubation time with the substrate compared with peroxidase conjugates (4 h compared with 20-30 min) and also the relative instability of the starch-iodine-penicillin substrate

for long-term keeping (more than 24 h), and in the presence of high concentrations of certain biological macromolecules (in particular, lipids). However, the simplicity, ready availability, and nontoxicity of this substrate, the production of the lactamase conjugate by a very simple conjugation technique (the glutaraldehyde method), and its stability (in our experiments the lactamase conjugate did not suffer any loss of enzyme activity during keeping for 18 months at 4°C) all give the hope that conjugates based on β-lactamase will be widely used for the construction of various immunoenzyme test systems.

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METHOD OF IDENTIFICATION AND ISOLATION OF ORGANS OF ENDOCRINE SECRETION IN MICE

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KEY WORDS: endocrine secretion; identification

Noninbred and inbred mice have become the animals most widely used in scientific research because of their small size, rapid reproduction and development, and low cost of feeding and maintenance. A very important advantage of mice as experimental animals is the small doses of biochemical, pharmacological, toxicological, and other preparations used experimentally, which are often very expensive. In particular, we chose mice for electron-autoradiographic experiments, mainly on the grounds of the cost of labeled compounds.

Organs of endocrine secretion were removed from mice to study the difficult problems of identification and isolation of these organs. This is due to the small size of the mice themselves, their very small body weight, and some features of the arrangement of their endocrine organs. No description of such an investigation could be found in the literature, and the methods used to discover and isolate the adrenals, pituitary, and thyroid gland are described below (Table 1).

TABLE 1. Parameters of Weight of Endocrine Organs in Mice

Organ	Weight	
	mg	mg/100 g body weight
Pituitary	1,2±0,18	6,45±0,21
Adrenals	2,43±0,36	10,71±0,87
Thyroid gland	3,22±0,43	13,25±0,52

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