DEVELOPMENT AND USE OF AN ELISA-BASED METHOD OF SPECIFIC IgG DETERMINATION FOR DIAGNOSIS OF PULMONARY ASPERGILLOSIS

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The widely distributed fungi of the genus *Aspergillus* are known as agents of pulmonary aspergillosis. Five different diseases caused by these microorganisms have been identified: invasive aspergillosis, aspergilloma, bronchial asthma, exogenous allergic alveolitis, and allergic bronchopulmonary aspergillosis [3]. The agent of four of the above-mentioned diseases is usually *Aspergillus fumigatus* (A.f.), and only exogenous allergic alveolitis is more frequently caused by *Aspergillus clavator* and *Aspergillus niger* [1, 3].

The differential diagnosis of aspergillosis is difficult. At present a whole range of laboratory methods is used for this purpose, including sputum culture, skin tests, and methods of serodiagnosis, namely: determination of total IgE, immunoprecipitation, and determination of specific IgE and IgG.

Commercial methods of determination of specific IgG to A.f. antigens have not yet achieved wide popularity, and this makes correct diagnosis more difficult.

We have developed a method of determination of specific antibodies of the IgG class to A.f. antigens on the basis of ELISA.

EXPERIMENTAL METHOD

The antigen consisted of a filtrate of a 7-day culture of A.f. (Warsaw Tuberculosis Institute strain, mixture of lines 1, 2, 3, 7, and 8), obtained in the Laboratory of Fungi and Flora of the Central Tuberculosis Research Institute, Ministry of Health of the USSR. After primary purification by diafiltration with immersed CX-1000 ultrafilters ("Millipore," USA), with peak screening threshold of 10 kD, the preparation was washed with 10 volumes of 0.1 M phosphate-buffered saline (PBS), pH 7.2-7.4, concentrated fivefold relative to the original volume, sterilized by filtration through pores 0.22 nm in diameter, and kept at 4°C in the presence of 0.02% sodium azide, 0.01% Thimerosal ("Sigma," USA), and 100 kU/ml trasylol ("Bayer," Germany).

Total serum IgE was determined by enzyme immunoassay [2]. The results were expressed in U/ml. Specific IgE to mold allergens were determined by the RAST method ("Pharmacia," Sweden). The results were expressed in protein units (PRU)/ml.

Sera from patients with aspergillosis and aspergilloma, used as the positive control, were obtained from the Laboratory of Fungi and Flora, Central Tuberculosis Research Institute. Ministry of Health of the USSR. High titers of precipitating antibodies to antigens of A.f. and other species of *Aspergillus* were determined in them by the double diffusion test and by counter immunoelectrophoresis. Serum for the negative control was obtained from normal individuals. Blood sera for determination of the limits of the concentration of IgG to A.f. were obtained from 138 patients with bronchial asthma, whose total IgE exceeded 500 U/ml, and were aged from 15 to 77 years (80 men and 58 women), patients of departments of allergology and pulmonology of the No. 57 General Hospital in Moscow. Specific IgE to a mixture of molds was detected in 78 patients at levels between 0.350 and 4.341 PRU/ml.

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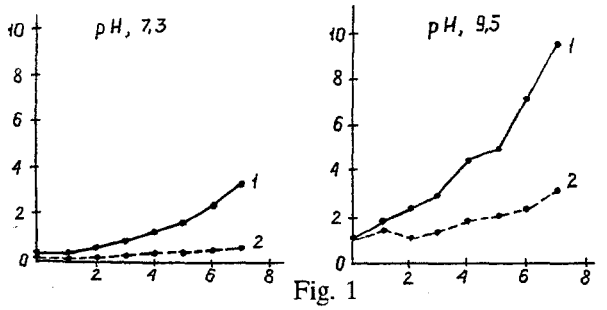

Fig. 1

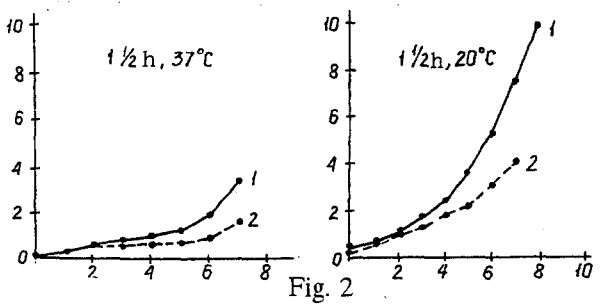

Fig. 2

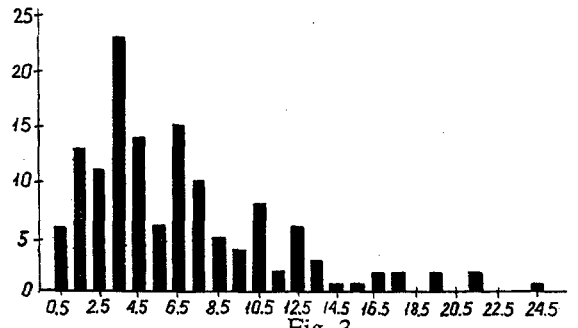

Fig. 3

Fig. 1. ELISA. Choice of optimal buffer for application of antigen. 1: (+)-serum, 2: (−) serum. Abscissa, log of concentration; ordinate, binding (in %).

Fig. 2. ELISA. Choice of time and temperature of incubation with serum. Legend as to Fig. 1.

Fig. 3. Distribution of concentrations of IgG to A.f. in bronchial asthma patients whose total IgE exceeded 500 U/ml. Abscissa, intervals of values of IgG to A.f.; ordinate, number of patients having IgG to A.f. within the given interval.

For ELISA the A.f. antigen was diluted with buffer in different concentrations, applied to microplates, and incubated for 16-18 h at 4°C. The wells were washed five times with 0.9% NaCl solution containing 0.1% Tween-20 ("Ferak," Germany), and incubated with sera diluted with PBS, containing 0.1% Tween-20 and 1% bovine serum albumin ("Serva," Germany) for 1-2 h at 37°C or at room temperature. After rinsing, rabbit antibodies to light chains of human immunoglobulins, labeled with horseradish peroxidase ("Dakopatts," Denmark), diluted with PBS-Tween (1:200-1:10,000), were introduced into the wells. The samples were incubated for 1 h at room temperature. The wells were washed as described above and a substrate mixture consisting of 0.002% H_2O_2 ("Fluka," Switzerland) and 0.08% orthophenylenediamine ("Sigma," USA) in 0.15 M citrate-phosphate buffer (pH 4.8-5.0), was applied. The optical density was determined on a "Multiscan MCC" photometer ("Flow Laboratories," Great Britain) at a wavelength of 492 nm. In the quantitative version of the method, calibration was carried out with a 1:2 dilution of the positive

control serum in PBS-Tween from the initial concentration of 1:100; the results were expressed in conventional units, and the antibody concentration in the positive control was taken to be 100 units.

The numerical results were subjected to statistical analysis by the Microstat ("Ecosoft") program.

EXPERIMENTAL RESULTS

When the ELISA method has been used for the diagnosis of aspergillosis in laboratories in the west, both an extract of a culture [12-14] and a culture filtrate of A.f. [6, 10, 11] has been used as antigenic material. In our method, a filtrate of a 7-day culture of A.f. was used.

Preliminary experiments with unpurified filtrate showed definite differences between sera from patients with aspergillosis and healthy individuals, however, the background of nonspecific binding differed greatly in different filtrates. In the case of high background values, crude purification of the cultural filtrate by diafiltration was used (with molecular weight threshold of over 10 kD). This procedure gave satisfactory results, and for that reason we did not resort to finer methods of purifying the antigen (salting out, gel filtration), such as were described in other publications [4, 5, 7, 8].

The choice of concentration of antigenic material for analysis was made separately for each filter. Optimal dilutions were between 1/200 and 1/500.

Binding of the antigen with the solid phase was found to depend strongly on the ionic strength and pH of the buffer with which the antigenic material was diluted (Fig. 1). Optimal adsorption of antigen on the surface of the microplate was obtained by using alkaline 0.1 M carbonate-bicarbonate buffer (pH 9.5), in agreement with methods used by other authors [4, 5, 9].

To choose the solid phase for analysis we tested microplates ("Flow") and strips ("Labsystems") made of polystyrene and microplates made of polyvinyl chloride ("Flow"). Polyvinyl chloride was shown to increase nonspecific binding strongly compared with polystyrene, and for that reason we rejected it for further use.

Some investigators used incubation for 1-1.5 h with serum at 37°C in their experiments [4, 5]. Unlike in theirs, in our experiments raising the incubation temperature sharply reduced the sensitivity of the method (Fig. 2); for the standard method we therefore chose incubation for 1.5 h at 20°C.

To conduct the analysis we used a standard dilution of the sera of 1/100 in 0.1 M PBS buffer.

The following optimal conditions for determination of IgG to A.f. by ELISA were thus established: 1) type of solid phase – polystyrene microplates ("Linbro") or strips ("Labsystems"); 2) buffer for dilution of antigen – 0.01 M carbonate-bicarbonate buffer (pH 9.5), incubation for 16-18 h at 4°C; 3) time and temperature of incubation with serum – 1.5 h at 20°C.

These conditions agree on the whole with previous methods, but they are adapted for concrete antigenic material and a concrete task.

A basic problem was standardization of the method. We selected sera for comparing the positive and negative controls. The negative control consisted of normal human sera with low optical density in the experiment. To prepare the positive control we used sera from patients with invasive aspergillosis and with aspergilloma, who had high optical density values. Later, the positive control was used to plot a calibration curve to determine values of IgG to A.f. To calculate the values, the linear region of the curve was chosen (Fig. 1). The results were calculated in conventional units ([+]-control = 100 conventional units).

To determine the limits of concentration of IgG to A.f. we tested 138 patients with atopic and mixed forms of bronchial asthma, with total IgE levels in excess of 500 U/ml. Of the 138 patients, 78 (59.5%) had specific IgE to mold allergens. Testing the hypothesis of normality of distribution of the results showed that they did not lie within the 95% confidence interval of probability of a normal distribution. The limiting concentration of IgG to A.f. was therefore chosen on the basis of the cumulative percentage of distribution: 92.5% of the patients tested had specific IgG to A.f. below the level of 16 conventional units (Fig. 3).

Raised values of IgG to A.f., from 16.7 to 36.4 conventional units, were found in nine patients with a tentative diagnosis of "bronchial asthma." All these patients had IgE levels above 1000 U/ml and had specific IgE to mold allergens. In five patients a detailed investigation confirmed the diagnosis of allergic bronchopulmonary aspergillosis.

LITERATURE CITED

1. V. I. Pytskii, N. V. Adrianova, and A. V. Artomasova, Allergic Diseases [in Russian], Moscow (1984).
2. O. A. Serdyuk, É. N. Tsytsykov, and R. G. Vasilov, Byull. Éksp. Biol. Med., No. 6, 722 (1989).
3. J. L. Elder and J. T. Smythe, Med. J. Aust., **1**, 231 (1967).
4. C. Harvey and J. L. Longbottom, Clin. Allergy, **16**, 323 (1986).
5. C. Harvey and J. L. Longbottom, Clin. Exp. Immunol., **62**, 231 (1986).
6. P. Haslam, A. Lukoszec, J. L. Longbottom, et al., Clin. Allergy, **6**, 227 (1976).
7. H. F. Kauffman and K. de Vries, Int. Arch. Allergy, **62**, 265 (1980).
8. H. F. Kauffman and K. de Vries, Int. Arch. Allergy, **62**, 252 (1980).
9. H. F. Kauffman, P. J. van Heyden, S. van der Laan, et al., Int. Arch. Allergy, **78**, 174 (1985).
10. J. L. Longbottom, Mykosen, Suppl. 1, 207 (1978).
11. J. L. Longbottom, Clin. Exp. Immunol., **53**, 354 (1983).
12. H. Schonheyder and R. Andersen, Int. Arch. Allergy, **74**, 262 (1984).
13. E. V. Wilson and V. M. Hearn, Mykosen, **25**, 653 (1982).
14. E. V. Wilson and V. M. Hearn, J. Med. Microbiol., **16**, 97 (1982).

EFFECT OF α-INTERFERON ON ENZYME LEVELS OF ADENOSINE METABOLISM AND MACROPHAGAL BACTERICIDAL ACTIVITY IN STAPHYLOCOCCAL INFECTION

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An important role in the pathogenesis of staphylococcal diseases is played by congenital and acquired pathologies of the phagocytic system [1, 5]. The writers previously studied the protective action of α-interferon (IFN) in experimental staphylococcal infection, due to activation of macrophages [6-8]. Meanwhile the mechanisms of stimulation of phagocytic cells by IFN in staphylococcal infection have still largely not been identified: changes in activity of the enzyme systems, the state of the intracellular metabolic processes, and the link with specific factors of immunity have not yet been adequately studied. The solution of these problems is an urgent task, for it is essential to an understanding of the mechanisms of formation of the antibacterial immune response in staphylococcal diseases, and also of its goal-directed regulation by immunomodulators. The functional activity of immunocompetent cells can be disturbed as a result of a change in activity of the enzymes of adenosine metabolism, namely 5'-nucleotidase and adenosine deaminase [9, 10]. The intracellular adenosine level determines the degree of maturation of receptors on the cells, and if present in excess, it causes inhibition of the differentiating and proliferative activity of the T and B lymphocytes, and also of macrophages [9-12].

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