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ENZYMIC INDICATOR OF IMMUNOBIOLOGICAL ACTIVITY OF ANTIGENS

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KEY WORDS: macrophages; acid phosphatase; antigen

A widely used indicator of the immunologic properties of different antigens, based on analysis of the serum antibody titer, is general in character and reflects only the end result of the immune process. Such an approach, if used during the production of vaccine preparations, cannot completely reflect all the diversity of immune reactions taking place in man and animals. At the present stage of development of immunology, the study of the action of different antigens on individual components of the immune system is very important. Accordingly, the development of a test system which would allow antigens used for vaccine production to be screened on separate populations of immunocompetent cells is an urgent task. An important aspect of this problem is the macrophagal component of the immune system. We know that an antigen undergoes processing in the macrophages, after which it is presented to lymphocytes [5]. In turn, when interacting with the antigen, the macrophage becomes activated, and this can be revealed by a number of methods based on determination of the respiratory burst, intensification of phagocytic and migration functions, and incorporation of fluorescent substances [4, 7]. However, these methods do not completely reflect the trigger function of antigens.

The activated macrophage accumulates and excretes a whole range of substances, including hydrolytic enzymes [4, 7, 8]. One such enzyme is acid phosphatase, activity of which is modified after stimulation of the macrophage and it can be used as an indicator of its activation [4].

The aim of this investigation was to develop a test system whereby the degree of activating ability of antigens of different nature in a culture of mouse peritoneal macrophages can he determined biochemically.

EXPERIMENTAL METHOD

To obtain peritoneal macrophages CBA mice aged 5-6 weeks were used. The mice were given an intraperitoneal injection of 5 ml of 3% peptone. On the 3rd day cells of the peritoneal exudate were harvested by flushing out the peritoneal cavity with 5 ml of medium 199, pH 7.2, containing fetal calf serum (5%), glutamine (0.03%), heparin (5 IU/ml), and antibiotics (penicillin and streptomycin, 100 IU/ml and 100 μg/ml respectively). A firm monolayer of macrophages was obtained by incubating $(3.5\text{-}4.0) \cdot 10^6$ peritoneal cells in 1.5 ml of medium for 2 h in 40-ml plastic Petri dishes at 37°C. After removal of the medium the adherent cells were washed twice with culture medium (without heparin, but with the addition of HEPES), and incubated in that medium after addition of the antigen (experimental samples) or without it (control samples) for 24 h in an atmosphere containing 5% CO_2, at 37°C. At the end of the incubation period the culture medium was removed, and the cell monolayer was washed once with Hanks' salt medium, containing 10% bovine serum, and then with buffered 0.15 M NaCl solution, pH 7.2. The

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TABLE 1. Incubation Mixture for Determination of Acid Phosphatase Activity

Ingredient	Exp	C_1, µl	C_s
Cell lysate	40	40	—
Triton X-100, 0.1% solution	—	—	40
NaCl, 0.15 M solution	50	50	50
Citrate buffer, 0.04 M, pH 5.0	40	80	40
Na p-nitrophenyl phosphate, 0.45% solution	40	—	40
NaOH, 0.02N solution	20	20	20

Legend. Incubation for 30 min at 37°C.

cells [(0.7-1.0) · 10^6 per dish] were lysed by the addition of 200 µl of a 1% solution of Triton X-100 to each Petri dish, followed by shaking for 15 min at room temperature. Next, to each dish 80 µl of a 0.15 M NaCl solution was added, the sample was thoroughly mixed, and the resulting solution of the cell lysate was transferred into microplanchets for determination of acid phosphatase activity. The ratio of the ingredients in the incubation medium for the experimental sample (Exp, the control sample of solution of lysate (C_1), and the control sample for the solution of substrate C_s, and also the order of addition of the ingredients, are given in Table 1. The optical density of the solutions was measured at 405 nm. Extinction, corresponding to activity of the enzyme, was found by the equation: $E_f = E_e - (E_l + E_s)$, where E_f is the extinction of the solution corresponding to enzyme activity, E_s the extinction of the experimental sample of solution of the lysate, E_l extinction of the control sample of solution of the lysate, and E_s the extinction of the control sample of the solution of substrate. The value of E_s found was compared with the calibration curve plotted for *p*-nitrophenyl (0.01-0.10 and 0.10-1.0 µmole/ml). Activity of the enzyme (in µmoles *p*-nitrophenol) of the experimental sample was expressed as a percentage of the control, taken as 100%.

Antigens of varied nature were used as activating agent: lipopolysaccharide of *Salmonella typhimurium* (Sigma), polysaccharide of *Salmonella typhi* (Vi-antigen) [1], glycoprotein [hemagglutinin of influenza virus, strain X-79 (H_3N_2)], isolated by the method in [6], a protein (genetically engineered human γ-interferon; Ministry of the Medical and Biological Industry, "Ferment" Research and Production Combine); peptide of γ-interferon and glycopeptide (murabutide), generously provided by V. A. Maiorov (Institute of Immunology, Ministry of the Medical and Biological Industry of the USSR).

The cells were stained with a 1% solution of trypan blue and counted in a Goryaev counting chamber.

EXPERIMENTAL RESULTS

In the initial stage of the investigation the concentration of cells required to obtain optimal results for determination of acid phosphatase activity was worked out. For this purpose the initial suspension of peritoneal cells, with differing concentrations, was seeded on Petri dishes and the cells were counted after culture for 2 and 24 h. As the results of this investigation showed, the number of adhering cells (macrophages) after 2 h of culture was just over 50% of the original concentration of cells in the samples. After culture for 24 h the number of viable macrophages was about 50% of the number of cells in culture after 2 h. Determination of enzyme activity in samples containing different numbers of cells, under conditions of activation by Vi-antigen, showed that optimal activity of the enzyme was present in the sample with the maximal concentration of macrophages, namely (0.7-1.0) · 10^6 cells in a petri dish. This number of macrophages in the Petri dish after 24 h of culture corresponded to (2.3-2.7) · 10^6 cells in 1 ml of the original peritoneal washings. The result of one typical experiment are given in Table 2.

In all the subsequent experiments to study the activating power of the antigens cells were used in the optimal concentration indicated above, namely (2.3-2.7) · 10^6 cells/ml. As the results of the investigation showed, not all the antigens used caused a change in acid phosphase activity, which could indicate absence of an activating effect on the cell. It was shown previously that acid phosphatase is a unique marker of activation of cells, including immunocompetent cells (lymphocytes and macrophages) [3, 4]. Under these circumstances activation was strictly temporary in character and depended on the dose of the activating agent. In the present investigation a maximal increase in enzyme activity was observed under the influence of lipopolysaccharide and polysaccharide (Vi-antigen) in a dose of 50 µg/ml (Fig. 1a, b), which corresponded to data obtained previously [4]. Human γ-interferon possessed only a weak effect, glycoprotein of influenza virus had an intermediate action (Fig. 1c, d). The optimal concentration of the protein and glycoprotein under these circumstances was 10 µg/ml. Agents with low molecular weight, namely the interferon peptide and murabutide, in optimal concentrations, had no effect on enzyme activity.

TABLE 2. Acid Phosphatase Activity in Samples with Different Concentrations of Cells, and Stimulated by Vi-Antigen

Number of macrophages in Peri dish, $\times 10^6$	Control		Vi-antigen		p
	E_s	%	E_s	%	
0,2±0,001	0,039±0,001	100	0,039±0,001	100	
0,6±0,006	0,080±0,002	100	0,130±0,006	162,5	<0,001
1,0±0,009	0,100±0,006	100	0,190±0,007	190	<0,001

Legend. Mean results from 5 tests shown.

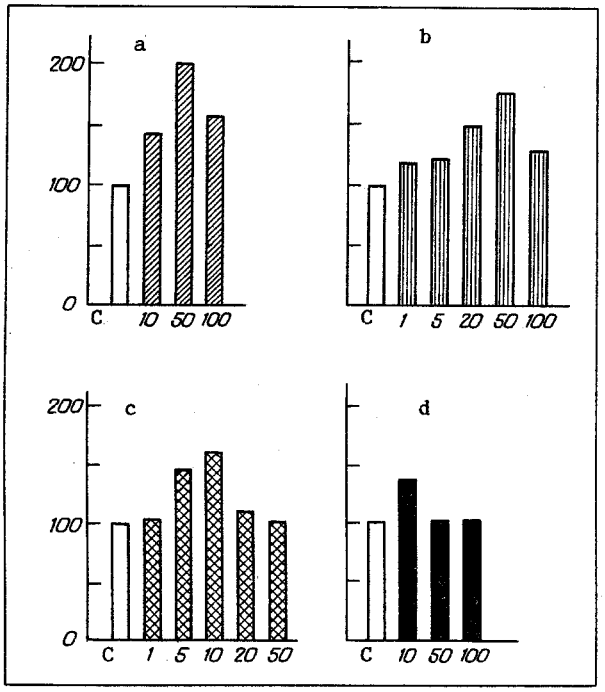

Fig. 1. Acid phosphatase activity in mouse peritoneal macrophages under the influence of various antigens. a) Vi-antigen; b) lipopolysaccharide; c) hemagglutinin of influenza virus; d) human γ-interferon. Abscissa, antigen concentration (in g/ml); ordinate, enzyme activity (in percent of control) (C).

In the test system now proposed, by contrast with that used previously [4], we greatly reduced the number of macrophages in culture. This was done by using 40-mm Petri dishes instead of 100-mm. In addition, the method of destroying the cell mass was modified: instead of mechanical destruction of the cells in a Potter homogenizer, we used a chemical method with 1% Triton X-100. Determination of activity of the enzyme in microplanchets enabled the optical density of the solutions to he measured on instruments for automatic specimen scanning. To simplify the calculation procedure, enzyme activity was expressed as a percentage of the control, taken as 100%. In this way high stability of the results was achieved.

The proposed test system can be recommended for use in laboratory practice for testing the immunobiological activity of various antigens.

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IMMUNE RESPONSE TO SYNTHETIC POLYSACCHARIDE–PROTEIN CONJUGATE

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KEY WORDS: synthetic polysaccharide; artificial antigen; enzyme immunoassay

The first attempt to create an artificial antigen by modifying natural carrier proteins by simple chemical compounds (haptens) was undertaken by Landsteiner in the 1930s [3]. Artificial antigens of carbohydrate nature were obtained by conjugation of natural antigenic polysaccharides, their synthetic fragments, or fragments obtained by their partial depolymerization, with proteins, and it was found that they are immunogenic, and that many of them possess protective properties [2, 5-7]. In the investigation described below a conjugate of the synthetic polysaccharide α-1,6-mannan with protein was obtained for the first time and the immune response of rabbits to this artificial antigen studied.

EXPERIMENTAL METHOD

A synthetic polysaccharide (SPS) containing an aglycone spacer with a free amino group, was α-1,6-mannan with $C_n \sim 10$, the reducing end of which was fixed in the form of 6-aminohexyl-glucopyranoside. The SPS was synthesized as described previously [1]. It was converted into the isothiocyanate derivative by treatment with thiophosgene and introduced into the reaction with amino groups of lysine residues of bovine serum albumin (BSA) [7]. According to the results of analysis the conjugate thus obtained contained 23% of carbohydrates and 71% of protein, corresponding to the addition of 11 moles of SPS to one mole of BSA. The BSA was obtained from "Serva" the thiophosgene from "Aldrich" and the SPS as described in [2]. The protein concentration was determined as in [4]. To determine the content of carbohydrates the conjugate was hydrolyzed (2 M CF_3COOH, 1 h, 120°C) and analyzed with the aid of a carbohydrate analyzer. Gel chromatography was carried out on a column (1.5 × 82 cm) with TSK HW-50S ("Merck," West Germany). Solution was carried out with 0.1 M phosphate buffer containing 0.15 M NaCl (pH 9.0). The elution curves were obtained with the aid of a UV-detector (280 nm) and a "Technicon" carbohydrate analyzer.

Chinchilla rabbits weighing 2-2.5 kg were immunized with SPS-BSA antigen (anti-SPS-BSA): first injection — 1 mg of antigen subcutaneously with Freund's complete adjuvant, followed one month later by the second injection — 1 mg of antigen intravenously. Bleeding was carried out on the 7th day after the second immunization, and the serum prepared from the blood was kept at −30°C or lyophilized. Antibodies to SPS were determined by the indirect method of enzyme immunoassay (EIA), at the end point of the reaction. Optimal doses of antigen were established by titration of the anti-SPS-BSA using different doses of antigen. The solid-phase carrier for immobilization of the antigen consisted of polystyrene plates ("Linbo," USA). All the operations were performed at room temperature. The antigen was dissolved in carbonate–bicarbonate buffer (pH 9.0) in a dose

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