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Rapid Determination of Anti-Enzyme Titer in Serum Using Whole *Staphylococcus aureus* Cells

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Abstract

Heat-killed, formalin-fixed *Staphyloccous aureus* (Cowan) cells are shown to be an excellent immunoadsorbent for quickly and accurately measuring the titer of antibodies directed against enzymes. The simple procedure is illustrated by following the course of immunization of a rabbit against mouse uridine 5′-monophosphate synthase. The antibody titer of a serum sample can be determined in roughly 1 h plus the time to perform the assays of enzyme (antigen) activity.

Index Entries: *Staphyloccus aureus*, use of in determination of antibody titer; anti-enzyme titer, determination of using whole *Staphyloccus aureus* cells; immunoadsorbent, solid-phase; uridine 5′-monophospate synthase; rapid determination of anti-enzyme titer; serum, rapid assay of anti-enzyme titer in.

Introduction

A major deterrent to raising antibodies against an enzyme is often the problem of developing a rapid and straightforward method for the quantitation of the level of antibody in the serum of an animal immunized against the enzyme protein. Antibodies can be detected in a variety of ways including by double diffusion in gels (*1*), by crossed immunoelectrophoresis (*2, 3*), by inhibition of enzyme activity because of the formation of binary immunoglobulin–enzyme complexes at the active site of the antigen (*4*, and references therein), by microcomplement fixation (*5*), or by radioimmunoassay using radiolabeled enzyme (*6*). Although each of these

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methods is reliable, each is hindered by being extremely cumbersome and/or insensitive, or by demanding considerable time and effort to establish an unfamiliar immunochemical method.

Double diffusion (*1*) often takes days to accomplish and requires relatively large amounts of antigen and antibody to produce a visible precipitin line. Immunoelectrophoresis (rocket, etc.) requires rather involved and specialized equipment (*2, 3*). The measure of antibody-produced inactivation of enzyme activity, although specific, is beset by two problems: (1) only antigen–antibody complexes in which the active site is disrupted can be detected, lowering the sensitivity of the assay, and (2) the optimal formation of antigen–antibody complexes is limited to a narrow ratio of reactants (i.e., the equivalence point). Complement-fixation (*5*) or radioimmunoassay (*6*) are, in many cases, fine alternatives; however, such methods seem unnecessary when one can measure the antigen in solution with great sensitivity by following its associated enzyme activity.

The primary requirement for a rapid and reliable immunoassay is an immunoadsorbent that will readily separate free antigen from antibody-bound antigen. This separation is probably best accomplished by the use of heat-killed, formalin-fixed *Staphylococcus aureus* (Cowan) cells (SAC cells) coated with Protein A (*7, 8*). SAC cells quantitatively precipitate all enzyme–antibody complexes and thus increase the sensitivity of the measurements of antibody levels. Although the double antibody technique is similar, it still is quite slow and subject to the equivalence problem. The recommended method is thus quite straightforward: the enzyme is briefly incubated with putative antiserum (or control serum) and commercially available SAC cells. After brief centrifugation, the remaining enzyme activity in the supernatant is measured. The entire procedure can be accomplished in essentially the time required for carrying out the enzyme assays, plus 1 h. This communication describes my experience using this simple procedure with the development of antibodies against the multienzyme uridine 5′-phosphate (UMP) synthase (*9*).

Experimental

Antiserum

A female New Zealand rabbit was injected with 150, 100, and 75 μg of homogeneous UMP synthase (*9*) on days, 0, 30, and 62, respectively. The purified antigen was mixed with 2 volumes of Freund's complete adjuvant and the mixture was homogenized by repeated evacuation and withdrawal through a syringe needle. The animal was immunized by several small subcutaneous injections about the neck and back. At indicated times the animal was bled by either drawing blood from the ear vein with vacuum, or dripping from a nicked vein. Tubes of blood were capped and left standing at room temperature for 1 h. Clots were separated from the glass by rimming the tubes with wood applicator sticks dipped in crystalline NaN_3 or merthiolate. The clotted blood was then chilled in the refrigerator overnight and the clots were removed by centrifugation, leaving clear serum in the supernatant. The serum was stored frozen (−20°C) in small lots.

Materials

Homogeneous (>98% pure) UMP synthase from Ehrlich ascites carcinoma was prepared as previously described (*9*). Freund's complete adjuvant and heat-killed, formalin-fixed SAC cells (Pansorbin) were obtained from Calbiochem. Prior to use the cells were washed several times with 50 m*M* Tris-Cl (pH 7.5), containing 10 m*M* Na_2 EDTA, 150 m*M* NaCl, 0.1% Nonidet P-40, and 0.01% NaN_3 (TESNA). The cells were resuspended at a concentration of 10% (w/v) in TESNA. Yeast glucose-6-phosphate dehydrogenase and rabbit anti-glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Company.

Enzyme Assays

Orotidine 5'-monophosphate (OMP) decarboxylase activity was determined as previously described (*9*). Activity is given as milliunits (nmol CO_2 formed/min) and represents the activity of UMP synthase. Glucose-6-phosphate dehydrogenase activity was measured at 22°C by following the increase in absorbance at 340 nm arising from the reduction of $NADP^+$. Reaction mixtures contained 50 m*M* Tris-Cl, pH 7.7, 0.8 m*M* glucose-6-P, 0.3 m*M* $NADP^+$, 10 m*M* $MgCl_2$, and an aliquot of enzyme in a total of 1.0 mL.

Immunoassays

Assays to determine antibody titer were performed in 1.5 mL microfuge tubes and were in a total of 0.5 mL of TESNA buffer. Each incubation contained 50 μg of non-immune carrier IgG (Sigma) and about 1 mU of OMP decarboxylase or 7 mU of glucose-6-P dehydrogenase activity. After the immune serum had been incubated with enzyme for 20 min at room temperature, 50 μL of washed SAC cell suspension was added and the mixtures were incubated for an additional 20 min. The bacteria were then pelleted by centrifugation in an Eppendorf Microfuge for 5 min, and aliquots of the supernatants were immediately assayed for enzyme activity.

Definition of Antibody Titer

Antibody titer is defined as the reciprocal of the volume of antiserum required to obtain a 50% removal of a fixed amount of enzyme activity obtained in a control immunoassay in which no putative antiserum was added. The dimension is defined as μL^{-1} and is analogous to the term "reciprocal of antibody dilution" (*10*).

Results and Discussion

In Fig. 1, the results of typical antigen–antibody titration curves for both rabbit anti-yeast glucose-6-phosphate dehydrogenase IgG fraction (Fig. 1A) and rabbit anti-UMP synthase serum are shown (Fig. 1B). It is evident that sufficient SAC cells were added in both experiments since total removal of enzyme activity was achieved with sufficient antibody. By varying the volume of SAC suspension with a constant amount of IgG (data not shown), it was determined that approximately

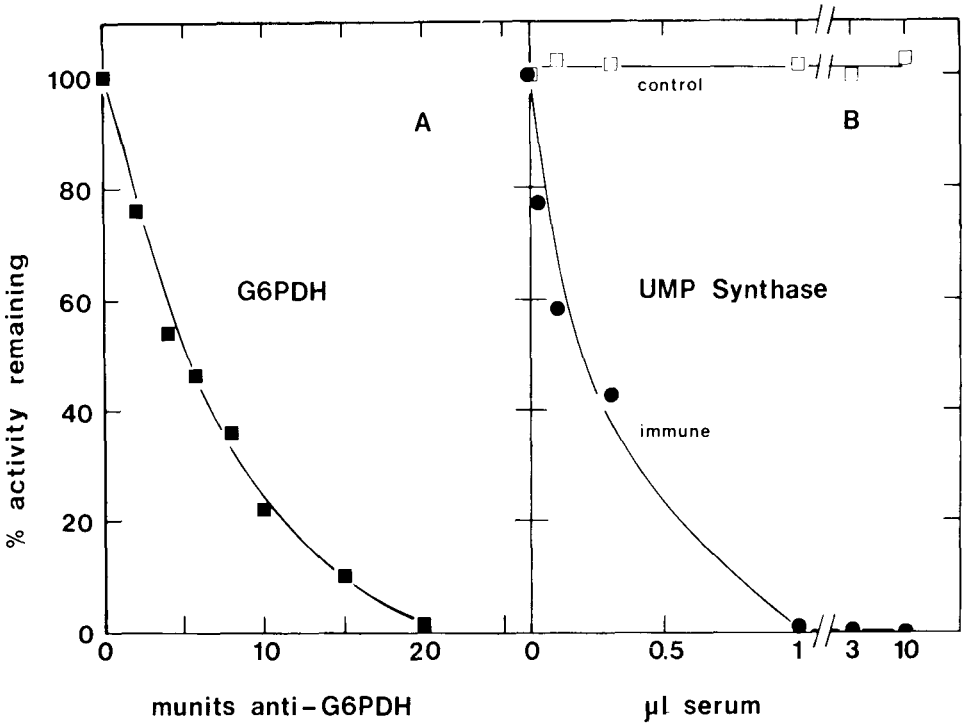

Fig. 1. Immunotitrations of glucose-6-phosphate dehydrogenase (G6PDH), *A*, and UMP synthase, *B*, as described in the Experimental section. One unit of anti-glucose-6-phosphate dehydrogenase is defined as the amount of antibody that precipitates one international unit of the enzyme. Immunoassays were performed as described in the Experimental section. As shown in panel *B*, the experiment was performed with pre-immune (□) or immune (●) serum, obtained 44 days after the initial injection of antigen.

50 µL of bacterial suspension would precipitate 100 µg of IgG, which corresponds roughly to an equivalent of 10 µL of serum. Using the procedure described here it was quite straightforward to follow the course of immunization of a rabbit against mouse UMP synthase.

Figure 2 shows a composite of antiserum titration curves obtained in the same manner as described above. The values of antiserum titer as well as the immunization and bleeding schedule are compiled in Table 1. As expected, initial immunization and one booster injection caused an initial low immune response, the third injection caused a further enhanced response, followed by a characteristic gradual decrease. This paper thus proposes an extremely simple, fast, and reliable method for the determination of the level of antibodies directed against an enzyme. The method employs SAC cells that have been used successfully for cell surface antigen isolation (*7*), isolation of cell-free translation products (*11*), radioimmunoassays (*8, 12, 13*), and isolation of polyribosomes containing nascent proteins (*14, 15*) to name a few.

The use of SAC cells to separate bound from free antigen is not novel; however, SAC cells did not receive mention among the methods for antibody determination described in the recent Volume 70 of *Methods in Enzymology* (*16*) for

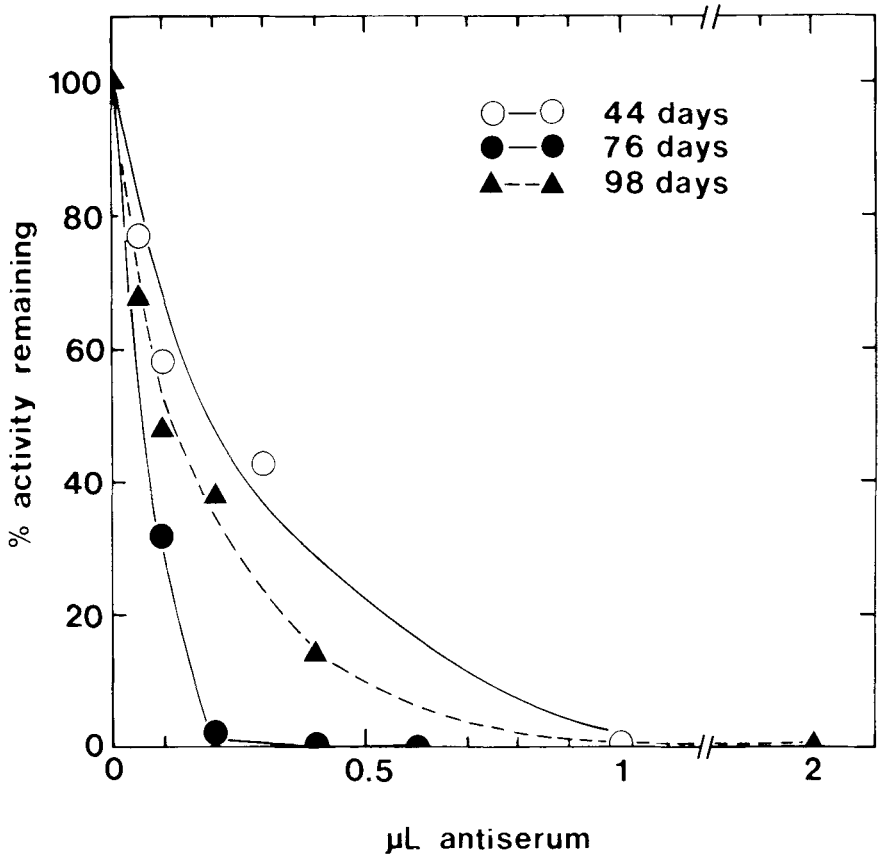

Fig. 2. Immunotitration curves observed using serum obtained from a rabbit 44 days (○), 76 days (●), and 98 days (▲) after the initial immunization. The complete immunization schedule is presented in Table 1.

TABLE 1
Progress of Immunization of a Rabbit with UMP Synthase

Day[a]	Event	Titer, [b] μL^{-1}
30	Booster	—
44	Test bleed	4.3[c]
62	Second booster	—
76	Test bleed	16[c]
91	Test bleed	12.7
98	Test bleed	8.0[c]

[a]After initial immunization.
[b]Reciprocal of antiserum required to remove 50% of UMP synthase activity (1 mU).
[c]Analyses are depicted in Fig. 2.

immunochemical methods.† Largely for that reason it seemed useful to describe my experience using this application of the remarkable affinity of SAC cells for the F_c portion of IgG (*18, 19*) to the task of measuring antibody titer rapidly. Not only is the present method faster than other standard immunochemical techniques presently used, it is much more sensitive for two reasons. First, since the antigen has enzymatic activity, the measurement of the amount of unbound antigen can be amplified to a theoretically unlimited extent by using sensitive radiochemical (as for UMP synthase) or spectrophotometric (as for glucose-6-phosphate dehydrogenase) assays. In this respect, the proposed method is similar to ELISA (enzyme-linked immunosorbent assay) techniques [see, for example, ref. (*20*)]. Second, all antibody species are recognized rather than just those that cause direct enzyme inactivation, since SAC quantitatively precipitates all antigen–antibody complexes (*7*) and is not limited by a narrow ratio of antigen/antibody for optimal precipitation.

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†During the preparation of this manuscript a method which describes the use of SAC cells to separate bound and free peptide hormones appeared in Volume 73 of *Methods in Enzymology* (17).

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