UNEXPECTED LOSS OF IMMUNOREACTIVITY OF A GLUCOSE OXIDASE-LABELED ANTIGEN IN A STEROID ENZYME IMMUNOASSAY SYSTEM

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Glucose oxidase-labeled 11-deoxycortisol was tested for immunoreactivity in an enzyme immunoassay system using a monoclonal antibody. The antigen was labeled by the N-succinimidyl ester method. It was found that the binding affinity of the label to the anti-steroid antibody markedly decreased during storage; the half-life time was ca. 4.5 d.

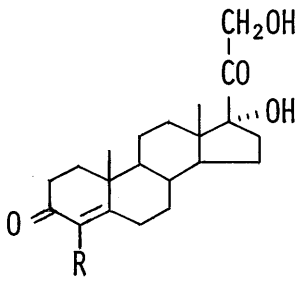
KEYWORDS enzyme immunoassay; enzyme labeling; 11-deoxycortisol; glucose oxidase; glucose oxidase-steroid conjugate; monoclonal antibody

Enzyme immunoassays of haptens have been developed with various enzymes. Glucose oxidase (GOD) is frequently used as an enzyme label. This enzyme catalyzes the oxidation of β-D-glucose to D-glucono-δ-lactone in the presence of molecular oxygen, yielding hydrogen peroxide; its activity can be measured by a colorimetry or fluorimetry in combination with horseradish peroxidase. Previously, we developed a testosterone enzyme immunoassay system using 3,3',5,5'-tetramethylbenzidine (TMB) as a safe chromogen.[1] We report here unexpected results in the immunoreactivity of a GOD-labeled antigen in a monoclonal enzyme immunoassay system for the determination of 11-deoxycortisol.

Enzyme labeling of 11-deoxycortisol was carried out by the N-succinimidyl ester method. A solution of the N-succinimidyl ester of 4-(2-carboxyethylthio)-11-deoxycortisol (CET) (14 μg) in dioxane (0.1 ml) was added at 0°C to a solution of GOD (EC 1.1.3.4: Aspergillus niger, 287 U/mg) (1 mg) in 0.05 M phosphate buffer (PB), pH 7.3 (0.2 ml), and the mixture was gently stirred at 4°C for 4 h. After addition of PB (0.7 ml), the resulting solution was dialyzed against cold PB (3 l) for 2 d. A 0.6 ml sample of the dialyzed conjugate solution was transferred to a test tube; the solution was stored at 4°C at a concentration of 500 μg/ml, adjusted with PB containing 0.1% gelatin and 0.9% NaCl (assay buffer).

The monoclonal anti-11-deoxycortisol antibody (CET-M8) used was that secreted from the hybridoma, S.CET.M8.1.1, which was derived from fusion of P3-NS1/1-Ag4-1 myeloma cells with spleen cells of BALB/c mice immunized with CET linked to bovine serum albumin. This antibody has an affinity constant of 2×10^{10} M^{-1} for 11-deoxycortisol, in the radioimmunoassay procedure.[2]

The immunoreactivity of the GOD-labeled antigen was tested 0, 3, 5, 6 and 10 d after the preparation of the label. The enzyme immunoassay procedure was carried out in duplicate in a glass test tube (10 ml) as follows: GOD-labeled 11-deoxycortisol (100 ng) in assay buffer (0.2 ml) containing 0.25% normal mouse serum was added to diluted CET-M8 (1:100000, 0.1 ml), and the mixture was incubated at 4°C for 4 h. Rabbit anti-mouse immunoglobulin G antiserum (0.1 ml) diluted 1:120 with assay buffer containing 0.3% ethylenediaminetetraacetic acid was added to the incubation mixture, and the solution was vortex-mixed, then allowed to stand at 4°C for 16 h. The resulting mixture was diluted with assay buffer (1.5 ml) and centrifuged at 3000 rpm for 10 min, and the supernatant was removed by aspiration. The immune precipitate was washed once with assay buffer (1.5 ml) and diluted with 0.05 M acetate-citric acid buffer, pH 4.2 (2 ml), containing 0.4 mM TMB, 3% dimethyl sulfoxide, 0.5 M glucose, and 25 nM horseradish peroxidase, vortex-mixed, and incubated at 37°C for 1 h. The reaction was terminated by addition of 0.5 M H_2SO_4 (2 ml). The absorbance was measured at 450 nm. An experiment using only the GOD label was carried out to obtain the total enzymic activity. For comparison, an 11-deoxycortisol assay system using β-galactosidase (β-GAL) as a label enzyme[3] and a



11-deoxycortisol: R=H
CET: R=S$(CH_2)_2$COOH

Chart 1

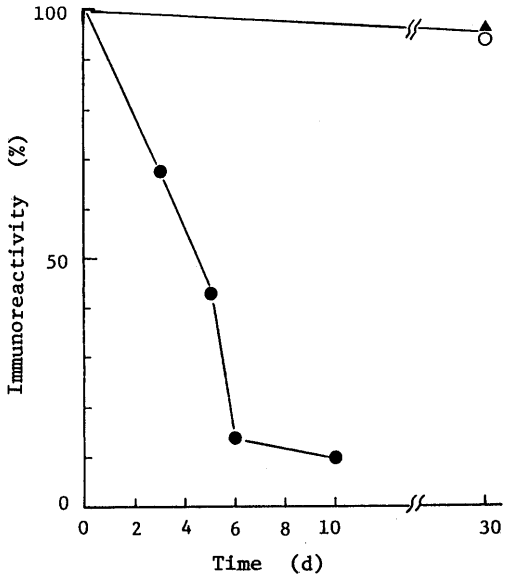


Fig. 1. Immunoreactivities of GOD-Labeled 11-Deoxycortisol (●) and Testosterone (○), and β-GAL-Labeled 11-Deoxycortisol (▲) with Anti-steroid Antibodies

testosterone assay system with a GOD label[1] were also tested. The results are shown in Fig. 1. The immunoreactivity was expressed for convenience as a percentage of that obtained immediately after the label preparation (time 0). It can be seen that the ability of the GOD-labeled 11-deoxycortisol to bind to CET-M8 is markedly decreased, compared to the reference systems: the half-life time is ca. 4.5 d.

At the present time, the reason for this decrease in immunoreactivity of the GOD label is not apparent. It seems unlikely that a conformational change of the GOD label takes place, which results in the steric hindrance between the steroid portion in the antigen and the antibody molecule, since the GOD-labeled testosterone was found to be intact under similar storage conditions. A possible explanation for this phenomenon is that the dihydroxyacetone chain of 11-deoxycortisol in the GOD label is oxidized, even on the surface of the macromolecule, by the enzyme as a substrate. The possibility cannot be excluded that the side chain is degraded by a nonenzymatic reaction catalyzed by neighbouring groups on the label molecule, although the β-GAL-labeled antigen was stable. Further studies are in progress to elucidate this problem.

Recently, immunosensor systems have also been developed using GOD. Thus, the present findings are important in the enzyme immunoassay and related fields. A similar phenomenon must be observed in the cases of some corticosteroids and drugs.

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