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COMMUNICATIONS

Rapid drug haptenization procedure: application to gentamicin and quinidine

CIARAN M. REGAN, *Department of Pharmacology, University College, Belfield, Dublin 4, Ireland*

A rapid and simple drug conjugation procedure which utilizes the heterobifunctional reagent *N*-hydroxysuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate is described. With this protocol two drugs of diverse structure and solubility have been successfully haptenized. Rabbits immunized with these conjugates yielded drug-specific antibodies as judged by a solid phase radioimmunoassay. Furthermore, using this technique, no cross-reactivity could be detected. Such haptens also elicited an immune response in mice thus further extending their potential for the production of drug specific monoclonal antibodies.

Enzyme-modified immunoassay (EMIT) and radioimmunoassay (RIA) are rapidly being adopted for the analysis of serum drug levels. These techniques are simple to perform yet have the specificity and sensitivity that is required. A limitation to the widespread use of these techniques is the requirement of drug-specific antibodies. Such antibodies are usually obtained by immunizing with the drug haptenized to a carrier protein. A procedure is described here which renders drug-protein complexes stable by the introduction of a covalent bond with the heterobifunctional reagent, *N*-hydroxysuccinimidyl 6-(4′-azido-2′-nitrophenylamino)hexanoate (NAP) (Das & Fox 1979; Taylor et al 1979).

Materials and methods

Drug haptenization. Unless otherwise stated, all reactions were carried out under dim red light. Initially the bovine serum albumen (BSA; Sigma Chemical Co.) carrier protein, in 50 mM sodium phosphate, pH 7·5 (10 mg ml^{-1}), was succinylated by making the solution 400 μM with NAP (Pierce Chemical Co.) which had previously been dissolved in dioxane. The final dioxane concentration never exceeded 6% (v/v). The reaction was stopped after 30 min by passing the solution over a G-25 Sephadex (Pharmacia Fine Chemicals) column which removed the untreated NAP. Gentamicin (0·5 mg mg protein^{-1}; Sigma Chemical Co.) or quinidine sulphate (0·1 mg mg protein^{-1}; Sigma Chemical Co.) was then added to the combined protein-containing fractions. The solution was then irradiated with a 250 W tungsten halogen lamp at a distance of 30 cm for 30 min. With more insoluble drugs, such as quinidine (10 mg ml H_2O^{-1}), the conjugation reaction could also be carried out in buffer containing 20% dioxane (see below). Following this step all subsequent manipulations were carried out under normal laboratory lighting. Unconjugated drug was removed from solution by a 24 h dialysis against 3 changes of Dulbecco's phosphate buffered saline, without Ca^{2+} and Mg^{2+} (DPBS, Dulbecco & Vogt 1954). The drug conjugates were then stored at −20 °C until required.

Conjugation of [^{3}H]quinidine to BSA. [^{3}H]Quinidine (sp. act. 753 mCi mmol^{-1}; Radiochemical Centre, Amersham) was conjugated to BSA (0·25 Ci g protein^{-1}) as described above. To ensure complete solubilization of the radiolabelled drug, the photoirradiation step was carried out in buffer containing 20% dioxane. Specific and non-specific binding was determined by trichloracetic acid (TCA) precipitation. Portions of conjugated [^{3}H]quinidine were made to 200

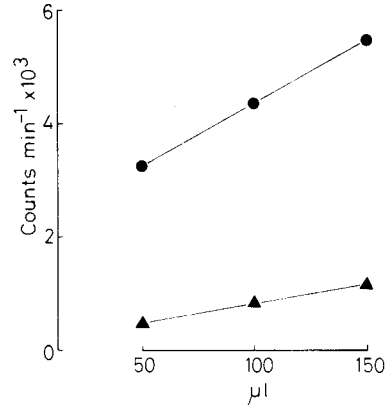

FIG. 1. Specific (●) and non-specific (▲) binding in aliquots of increasing volume of [^{3}H]quinidine conjugated to BSA.

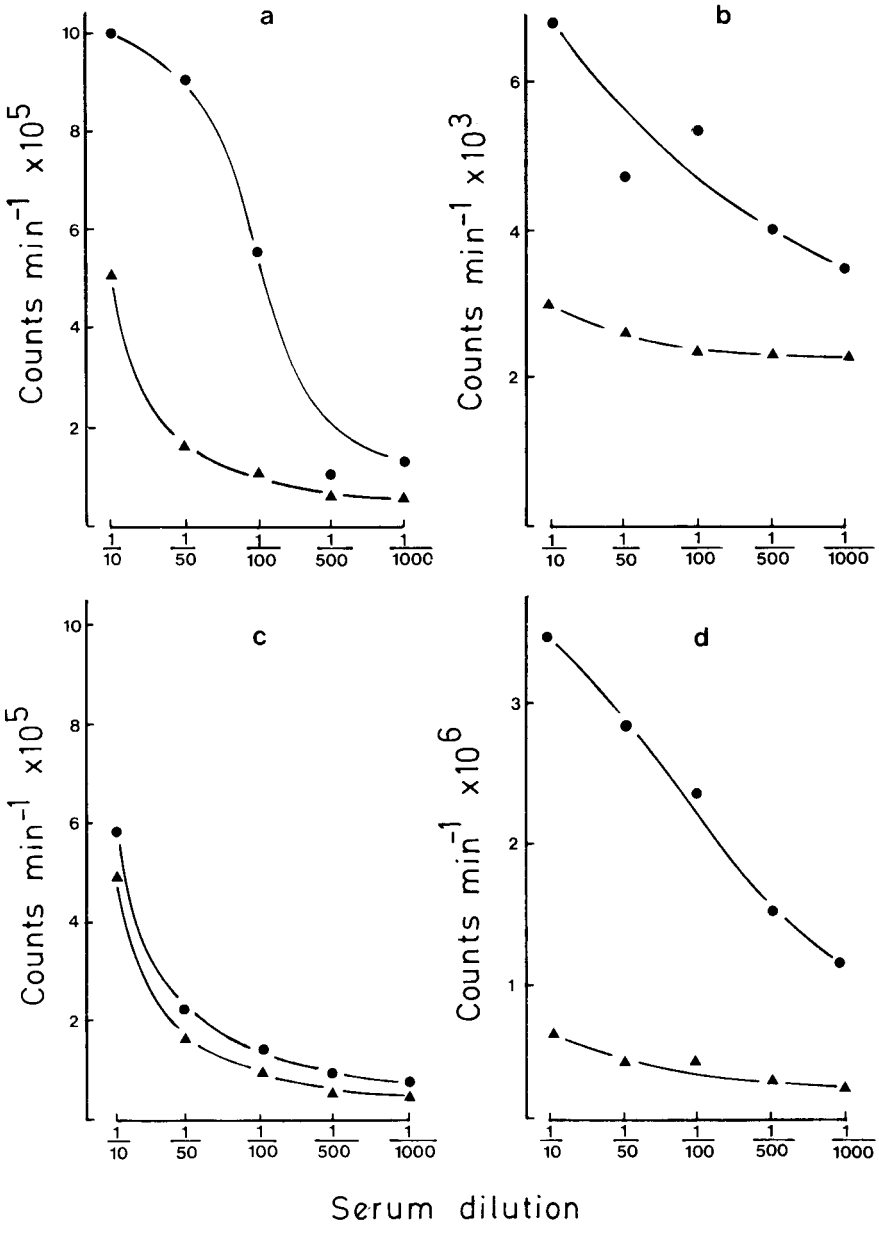

FIG. 2. Binding of serial dilutions of (a) rabbit anti-gentamicin antisera to gentamicin-coated wells; (b) rabbit anti-quinidine antisera to quinidine-coated wells; (c) rabbit anti-quinidine antisera to gentamicin-coated wells and (d) mouse anti-gentamicin antisera to gentamicin-coated wells. The circles (●) represent immune sera and the triangles (▲) pre-immune sera. The variation in counts min^{-1} bound is due to the half-life state of the ^{125}I-goat anti-rabbit second antibody.

μl with water followed by the addition of 100 μl of a BSA solution (100 mg ml^{-1}) to facilitate protein precipitation. 300 μl of a 10% aqueous TCA solution was then added and after mixing the tube was maintained at 0 °C for 30 min. The precipitated protein was collected by centrifugation (12 000*g* 1 min). After washing in 5% aqueous TCA the pellet was solubilized overnight in Soluene-350 (United Technologies Packard) and both it and the supernatant were counted by liquid scintillation.

Production of polyclonal antisera to drug conjugates. Rabbits were immunized, by sub-scapular injection with 1 mg of drug conjugate mixed with an equal volume of Freund's complete adjuvant (Miles Laboratories Ltd.) on days 1 and 7. The rabbits were re-immunized on day 21 with an intravenous injection of 100 μg drug conjugate. The sera were collected one week later. Mice were immunized in a similar fashion by intraperitoneal injection on days 1, 7 and 14 and intravenous injection on day 21. The antisera were collected 3 days later.

Solid-phase radioimmunoassay. Amounts (300 μl) of the drug to be assayed were allowed to bind to absorbent polystyrene wells (Immulon, Dynatech Laboratories Ltd.) at a concentration of 1 mg ml^{-1} DPBS for 30 min. After incubation the wells were washed with DPBS containing 1 mg ml^{-1} haemoglobin (Sigma Chemical Co.) to reduce non-specific binding. Serial dilutions of the antibody raised to the drug conjugate were then added to the wells and incubated for 30 min. Following this the wells were again washed as previously described and 300 μl of ^{125}I-goat anti-rabbit antibody (10^6–10^7 counts min^{-1} ml^{-1}; sp. act. 7 μCi μg^{-1}; New England Nuclear) added and incubated for 30 min. After washing the wells were γ-counted. All assay procedures were carried out at room temperature (20 °C).

Protein estimations were by the procedure of Lowry et al (1951) and, unless specified, all reagents were of the highest grade commercially available.

Results and discussion

The two drugs selected for conjugation were quinidine and gentamicin because of their diverse structure and respective cardiotoxic (Heissenbuttel & Bigger 1970) and nephrotoxic (Gary et al 1976) side effects. Furthermore, gentamicin can display erratic serum levels which require constant monitoring (Kay et al 1974). Thus these two compounds would ideally demonstrate the broad specificity of the conjugation procedure and are also amenable to a rapid assay utilizing specific antibodies. The covalent nature of this conjugation procedure could be demonstrated for quinidine as a tritiated form of the drug was available. Fig. 1 clearly demonstrates radiolabelled quinidine to remain conjugated to the carrier protein despite rigorous TCA precipitation which would easily disrupt non-covalent binding of drugs to plasma proteins. Fig. 2 clearly demonstrates the ability of NAP to conjugate gentamicin (a) and quinidine (b) to BSA in a fashion that can elicit an immune response. Furthermore these antisera can readily distinguish between different drug conjugates (Fig. 2c). The antisera also have significant titres and could be routinely diluted 1 : 50 to 1 : 100 without loss of their discriminating power, an important aspect in the development of routine immunoassay kits. The problem of batch variation and size is usually overcome by the use of monoclonal antibodies. These have the required specificity and can be produced in a continuous and unvarying supply. With this in mind, the effectiveness of these drug conjugates in eliciting an immune response in mice has also been demonstrated (Fig. 2d).

Thus this procedure clearly conjugates drugs and no loss of immunostimulatory activity was noted in haptens after prolonged storage at −20 °C or in a lyophilized state.

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