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THE DEVELOPMENT OF AN ELISA-ASSAY FOR SEMI-QUANTITATIVE DETECTION OF DIHYDROGRIESENIN, A SESQUITERPENE LACTONE FROM *GEIGERIA*

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ABSTRACT

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Certain species of *Geigeria* contain sesquiterpene lactones which cause vomiting disease in sheep. Dihydrogriesenin (DHG), a sesquiterpene lactone from *G. aspera*, contains an α-methylene function which can spontaneously react with thiol groups on proteins to form a covalent adduct. A specific antiserum against a DHG-protein adduct can be used to determine the fate of DHG in poisoned animals. The preparation of such an antiserum is reported in this paper.

DHG was reacted with cysteine and subsequently coupled to serum albumin using the carbodiimide reaction. When rabbits were immunized with one such conjugate (DHG-bovine serum albumin), it was found that the carrier determinants were immunodominant. A DHG-specific anti-serum of sufficient (ELISA) titre could, however, be obtained by alternating serum albumin carriers for DHG in booster immunizations. The ELISA antigen-antibody reaction could be inhibited by prior reaction of the antisera with cysteinyl-DHG in solution.

INTRODUCTION

Sesquiterpene lactones are currently being investigated for a variety of reasons (Rodriques, Towers & Mitchell, 1976). Some of the properties of these components are: antitumour activity (Hall, Lee, Mar & Starnes, 1977), antihyperlipidemic activity (Hall, Lee, Starnes, Muranka, Sumidy & Waddell, 1980), histamine liberation (Christensen & Rasmussen, 1980), inhibition of DNA synthesis (Woynarowski & Kanopa, 1982), inhibition of cardiac function (Pickman, Elliot & Towers, 1981), antifeedant activity (Doskotch, Fairchild, Huang, Wilten, Beno & Christoph, 1980), allelopathic effects (Kanchan & Jayachandra, 1979), inhibition of respiration (Van Aswegen, Potgieter & Vermeulen, 1982; Hall, Lee & Eigebaly, 1978), anti-inflammatory action (Hall, Starnes, Lee & Waddell, 1980), irreversible inhibition of certain glycolytic enzyme activities (Gaspar, Potgieter & Vermeulen, 1986) and inhibition of histamine release induced by compound 48/80 (Gaspar, Verschoor & Vermeulen, 1986).

The presence of an exocyclic α-methylene function conjugated to a γ-lactone is a primary requirement for biological activity of the toxic sesquiterpene lactone (Kupchan, Fessler, Eakin & Giacobbe, 1970). It is known that α-methylene-γ-lactone functions react with thiol groups on proteins—by Michael addition—to form covalent adducts (De Borosjenö, 1973). Dihydrogriesenin (DHG) is a sesquiterpene lactone containing the α-methylene-γ-lactone functional group. It is isolated from *Geigeria*, a plant species commonly known as vomiting shrub and the causative plant of vomiting disease in sheep (Steyn, 1943). Besides DHG, a variety of sesquiterpene lactones has been isolated from *Geigeria* species (e.g. Vogelzang, Vermeulen, Potgieter & Strauss, 1978; Vermeulen, Vogelzang & Potgieter, 1978).

In this paper, a method is described for the preparation of antiserum against DHG coupled to various serum albumins.

MATERIALS AND METHODS

Preparation of immunogen

Dihydrogriesenin was isolated from *G. aspera* as previously described (Vermeulen *et al.*, 1978). A solution of DHG (4,8 mmol) in methanol (25 mℓ) was stirred into a solution of cysteine (5,8 mmol) in 70 % methanol (25 mℓ) at room temperature. The reaction product solidified from the solution within 20 min. Methanol (50 mℓ) was added to the reaction mixture, which was then stirred for a further 2 h at 50 °C. A product was obtained in 80 % yield after filtration and washing with methanol, which appeared pure on tlc (Silica gel 60, Merck) developed with n-butanol:acetic acid:water 3:2:1. A recrystallized sample with m.p. 202–205 °C was obtained from water for structural characterization. Elemental analysis, infrared and ultraviolet spectroscopy and nuclear magnetic resonance data were in agreement with the structural properties assigned to cysteinyl-DHG (Fig. 1).

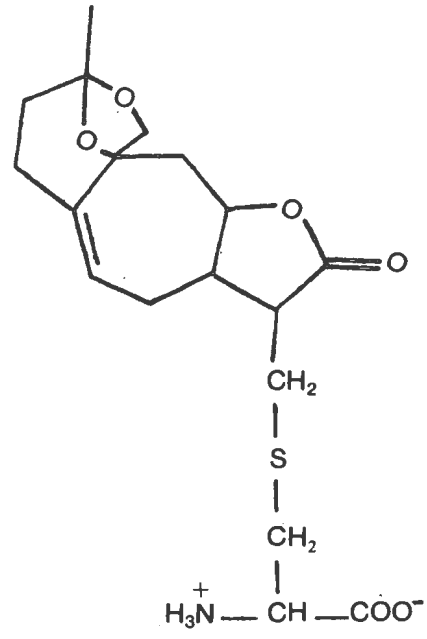

FIG. 1 Structure of cysteinyl-dihydrogriesenin

A solution of N-ethyl-N′-(3-dimethyl-aminopropyl)-carbodiimide. HCl (EDC, 2,6 mmol) in water (2,5 mℓ) was added to a solution of cysteinyl-DHG (0,13 mmol) and serum albumin (100 mg) in water (5 mℓ). After stirring for 1 h at room temperature, the mixture was dialysed and lyophilized to yield the immunogen. Epitope densities of the immunogens were determined by difference spectrophotometry at 242 nm between the conjugate and the relevant serum albumin. The difference spectrum revealed a peak at 242 nm, which presumably relates to the absorbance at 238 nm of DHG for which a molar extinction coefficient of $1,5 \times 10^3$ was determined. Conjugates with epitope densities, ranging

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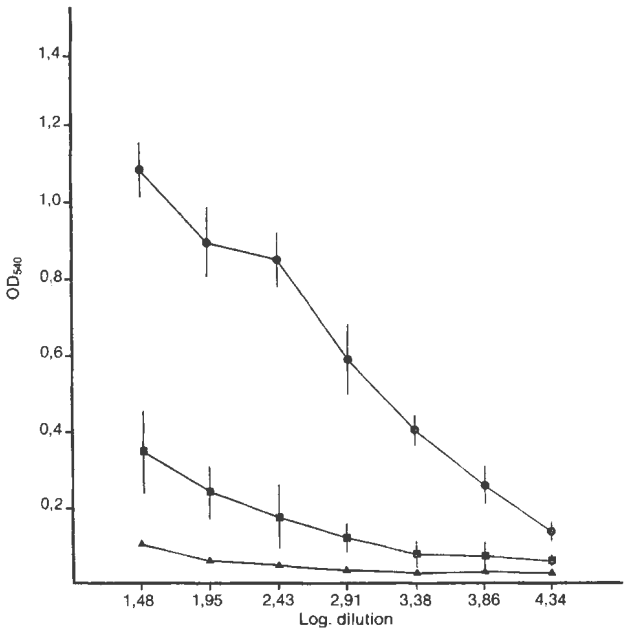

FIG. 2 Anti-DHG serum titre determination by ELISA

●–● Response of antisera (n=5) against DHG-rabbit serum albumin

■–■ Response of antisera (n=5) against rabbit serum albumin

▲–▲ Response of non-immune serum (n=1) against DHG-rabbit serum albumin

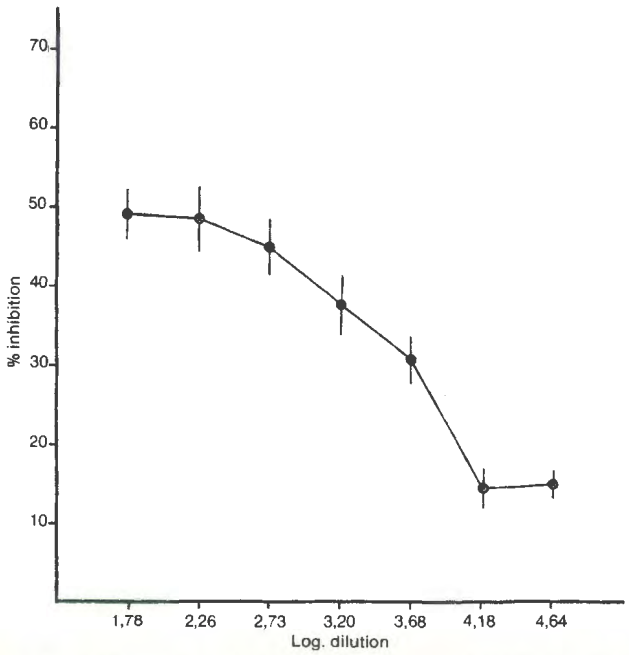

FIG. 3 Inhibition of anti-DHG serum with cysteinyl DHG. Each point represents triplicate values, using the antiserum of highest titre.

between 10 and 20 mol DHG/mol carrier albumin, were used for this study.

Immunization

Six-week-old Balb/C mice were immunized intradermally with DHG human serum albumin (60 μg) in Freund's complete adjuvant.* DHG-dog serum albumin (30 μg) and DHG-human serum albumin (30 μg) were

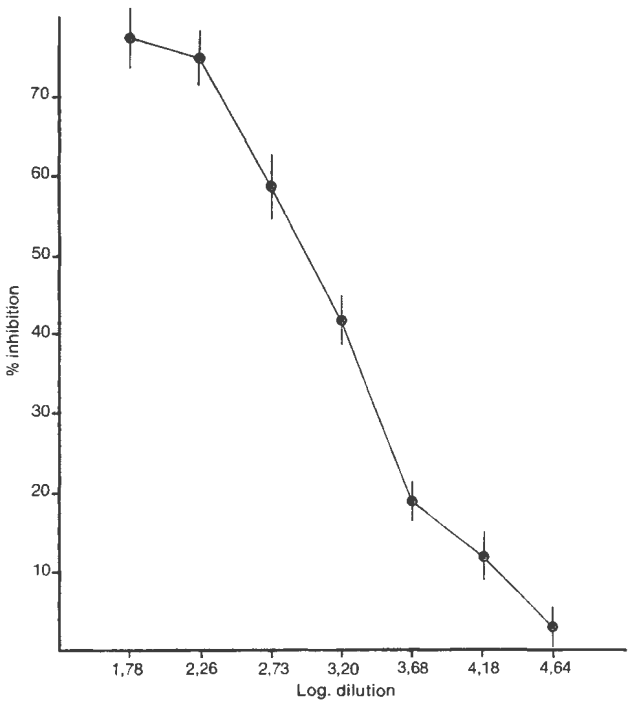

FIG. 4 Inhibition of affinity purified anti-DHG serum with cysteinyl-DHG.
Each point represents triplicate values, using the antiserum of highest titre.

alternated in subsequent boosters, which were subcutaneously administered at 4-week intervals. The animals were bled 7 days after the last booster injection. A control group was immunized with saline and adjuvant.

Titre determination

DHG specific antibodies were detected by an enzyme-linked, immunosorbent assay (ELISA). The antigen solutions of DHG-rabbit serum albumin and unconjugated rabbit, dog and human serum albumins were each dissolved in 0,05 M glycine—0,1 M NaCl, pH 2,5 at 1–10 μg/mℓ. The pH was adjusted to 7,0 with solid Tris (Conradie, Govender & Visser, 1983). Aliquots of 100 $\mu\ell$ of the antigen solutions were used to coat the wells on microtitre plates*, incubated at room temperature for 2 h with gentle shaking and washed with 0,05 M Tris, 0,1 M NaCl, 0,05 % Tween 20, pH 8,0 (TST) and distilled water. The remaining protein binding sites were blocked by incubation with 200 $\mu\ell$ of 3 % bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 1 h at room temperature with gentle shaking. The antigen-coated wells were dried under vacuum after the excess solution was removed. Aliquots (50 $\mu\ell$) of immune and control sera dilutions, prepared in 1 % BSA/PBS, were transferred to the antigen-coated wells, incubated for 1 h at room temperature, the excess solution being aspirated and the wells washed with TST and 1 % BSA/PBS. A 1:1000 dilution of horse radish peroxidase-conjugated rabbit anti-mouse IgG (Miles-Yeda) was prepared in 1 % BSA/PBS, aliquots (50 $\mu\ell$) transferred to the wells and incubated at room temperature for 45 min. The wells were washed with 1 % BSA/PBS, dried, and incubated with aliquots (50 $\mu\ell$) of the substrate solution (o-phenylenediamine, 1 mg; hydrogenperoxide, 30 %, 0,5 mℓ and citrate buffer 0,1 M, pH 4,5, 1 mℓ). The enzyme reaction was monitored at 450 nm in a Titertek Multiskan MC after 20 min.

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* Linbro Scientific, Hamden, Connecticut

Inhibition of the antigen-antibody reaction

The ELISA procedure described above was also used to measure the inhibition of the binding of antibodies to antigen-coated wells. Whole and affinity purified antisera were absorbed with equal volumes of 0,043 mM DHG-Cys in 1 % BSA/PBS before transferrance to the antigen-coated wells.

Affinity purification of antisera

CH-Sepharose 4B* (1 g) was washed with 0-5 M NaCl on a sintered glass filter. The ligand solution—0,3 μmol of equal amounts of human and canine serum albumin in distilled water (4 mℓ), pH 6,0—was added to the gel and gently mixed. This was followed by drop-wise addition of an EDC solution (2 mmol in 4 mℓ of distilled water, pH 6,0) and gentle mixing for 18 h at room temperature. Unbound protein was removed by washing with carbonate buffer (0,1 M, pH 8,3), acetate buffer (0,1 M, pH 4,0) and distilled water. The immunosorbent thus prepared was equilibrated in PBS prior to use.

Removal of carrier-specific antibodies was done by adding one volume of immuno-adsorbent to 1,2 volumes of antiserum, gentle mixing for 1 h at 37 °C and subsequent centrifugation at 8000 *g* for 5 min.

Results and Discussion

Synthesis of immunogens

Cysteinyl-DHG was synthesized in good yield with the structure, as shown in Fig. 1, determined by physical methods. It exhibited a lower R_f on tlc than DHG, presumably because of the polar moieties of the cysteine. The use of cysteine as hapten spacer on the protein carrier had a dual advantage: firstly, surface charge on the carrier molecule remains unchanged upon conjugation with the hapten, thereby retaining important chemical properties of the carrier such as iso-electric point, gross conformation and water solubility; secondly, the reactivity of the α-methylene group of DHG towards thiol groups on exposed tissue proteins is thought to be directly involved in incurring the observed tissue damage in poisoned animals, or indirectly by some immune mechanism (Pienaar, Kriek, Naude, Adelaar & Ellis, 1973). Antibodies against cys-DHG could therefore be used to determine the fate of DHG in poisoned animals, should it become bound to proteins and/or peptides.

Preparation of anti-DHG serum

Anti-DHG serum was raised in animals by immunization with DHG conjugated to serum albumin. A preliminary investigation, using rabbits as experimental animals, revealed a negligible anti-DHG response in comparison with the response against the carrier albumin. The ratio of anti-DHG to anti-carrier titre was significantly increased by alternating different serum albumins in the booster injections (D. C. Barnard, 1980, unpublished results). In this study, where Balb/C mice were used as experimental animals, anti-DHG ELISA titres of up to $2{,}2 \times 10^4$ were obtained against DHG-rabbit serum albumin as antigen after 3 booster injections (Fig. 2).

Inhibition studies and affinity purification

As is evident from Fig. 2 a considerable signal is generated from reaction of the immune serum to unconjugated rabbit serum albumin, presumably because of the presence of cross-reactive anti-carrier antibodies. Accordingly, inhibition of the ELISA-anti-DHG signal by prior incubation of antiserum with cysteinyl-DHG before incubation with DHG-rabbit serum albumin-coated wells could be effected to a maximum of only 50 %, with optimal values in the highest antiserum concentration range (Fig. 3). This prompted us to purify the antisera by affinity chromatography with human and canine albumins as affinity ligands. The success of this method is illustrated by the significant increase in specificity of the purified antiserum, as almost 80 % inhibition of the anti-DHG-rabbit serum albumin ELISA signal could be inhibited by prior incubation of affinity-purified antiserum with cysteinyl-DHG (Fig. 4).

* Pharmacia Biotechnology, Uppsala, Sweden

Conclusion

A specific antiserum has been developed against cysteinyl-DHG, the cysteinyl derivate of a sesquiterpene lactone isolated from *Geigeria*.

Removal of anti-carrier antibodies from the antiserum by affinity purification increased the specificity of the antiserum to such an extent that its ELISA signal against DHG-rabbit serum albumin could be inhibited by up to 80 % by prior incubation with cysteinyl-DHG. This system is now ready for application to explore the fate of DHG in poisoned animals.

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