0022-202X/79/0073-0250$02.00/0
THE JOURNAL OF INVESTIGATIVE DERMATOLOGY, 73:250–255, 1979

Vol. 73, No. 3
Printed in U.S.A.

The Role of Enzymatic Coupling of Drugs to Proteins in Induction of Drug Specific Antibodies

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Isoniazid and hydralazine were covalently crosslinked by epidermal transglutaminase to serum and cell nuclear proteins. Albumin and nucleohistones were excellent acceptors of these drugs in serum and nuclear extracts, respectively. Drug-albumin and drug-histone conjugates were highly antigenic in rabbits within 4 weeks, eliciting drug-specific antibodies in all immunized animals. A radioimmunoassay for antibodies to isoniazid was developed, using a standard drug-albumin conjugate.

The methods described here of enzymatic crosslinking of drugs to proteins facilitate studies of drug-induced hypersensitivity in animals and man.

Immune reactions to drugs are frequently associated with the development of circulating drug-specific antibodies; current methods of quantitating these antibodies, although sensitive, are cumbersome and difficult to standardize.

Lorand, Campbell, and Robertson observed in 1972 [1] that 2 drugs capable of inducing drug-specific antibodies in man, isoniazid (INH) and hydralazine (HYD) were excellent substrates for plasma transglutaminase. This enzyme ordinarily mediates covalent crosslinking between glutamine and lysine residues of fibrin, but can easily substitute small primary amines, such as INH or HYD for the lysine moiety of the reaction. Lorand, Campbell, and Robertson showed that both fibrin and serum albumin could be covalently labeled with drug in the presence of transglutaminase. Such accidental crosslinking, if it occurred *in vivo*, could create highly antigenic compounds capable of causing immune or autoimmune reactions, such as have been described following administration of INH [2–4].

In order to study further the enzymatic crosslinking of INH and HYD to tissue proteins and the immunologic consequences of such crosslinking, the findings of Lorand, Campbell, and Robertson with plasma transglutaminase and serum proteins were extended to include an intracellular enzyme, epidermal transglutaminase, capable of covalently crosslinking drug and nuclear constituents. Serum albumin and cell nuclear proteins were investigated as acceptors for INH and HYD. Drug-protein conjugates of known molar ratio were used to induce drug-specific antibodies in rabbits, and a radioimmunoassay using standardized ^{3}H-INH-albumin conjugates was developed.

MATERIALS AND METHODS

^{3}H-isoniazid (^{3}H-INH) with a specific activity of 5.1 mCi/mg was purchased from Amersham (Arlington Heights, IL). A 10 mM solution of cold INH was mixed with ^{3}H-INH to a specific activity of 1.25×10^{-4} mCi/mM. Hydralazine hydrochloride, rabbit and human serum albumin, lysine-rich histones, purified native calf thymus DNA, RNAase-free sucrose and enzyme grade Trizma base were obtained from Sigma (St. Louis, MO), and precast Ouchterlony plates from Hyland Laboratories (Oakland, CA).

Extractable nuclear antigen (ENA) and deoxyribonucleoprotein were prepared from fresh calf thymus according to the methods of Allfrey, Littau, and Mirsky [5] and Tan [6]. Immediately prior to use they were dialyzed into TBS and the protein concentration adjusted to 2 mg/ml. The presence of immunologically intact Sm and RNP antigens within the ENA preparation were verified by Ouchterlony diffusion against known positive sera.

Epidermal transglutaminase, purified from bovine epidermis as previously described [7], had an activity of 86.1 amine-incorporating units/ml. Protein concentrations were determined [8], using bovine albumin as standard.

Serum and nuclear proteins (histones, DNA, ENA, deoxyribonucleoprotein) were evaluated for their relative ability to incorporate ^{3}H-INH in the presence of transglutaminase. A timed 60 min incorporation study was carried out; duplicate assays were performed, using the same batch of enzyme, in an assay medium containing 1 mg nuclear protein, 10 mM Ca^{++} and 0.1 ml purified transglutaminase in a total volume of 0.85 ml at 37°C, pH 7.5. Calcium chloride was added to initiate enzymatic coupling and the reaction was stopped from 0–60 min with excess EDTA. Following exhaustive dialysis against TBS to remove unbound INH, 50 μl aliquots of the reaction mixture were counted in duplicate in a Beckman Liquid Scintillation Counter. Specific activity of the conjugates was calculated by proportionality against the starting solution of INH.

For gel filtration, Sephadex G200 was obtained from Pharmacia (Uppsala, Sweden) and was performed under standard conditions at pH 7.5, 25°C with TBS as eluting buffer. The column was precalibrated with Blue Dextran, γ-globulin, RSA and Cytochrome C (Sigma).

Hydralazine and isoniazid were conjugated with transglutaminase for 120 min to HSA, RSA and lysine-rich histones for injection into experimental animals. The crosslinking medium contained drug at 10 mM (final concentration); transglutaminase, 0.25 ml (specific activity 11.4 AIU/ml); carrier protein, 4 mg/ml (avg mw histones = 20,000). Unreacted drug was removed by dialysis against TBS − 1 mM EDTA and extent of drug conjugation measured. Unreacted drug was removed by dialysis against TBS − 1 mM EDTA, and extent of INH conjugation measured as above for isoniazid. Conjugation of hydralazine to protein was quantitated by measuring the change in absorbance at 275 nm and comparing this with a standard solution of hydralazine in TBS [9]. The contribution due to protein absorption was measured separately and subtracted. Pairs of New Zealand white rabbits received initial foot pad injections with 1.5 mg conjugate in complete Freund's adjuvant. They were given booster injections of 0.75 mg emulsified in incomplete adjuvant every 2 weeks thereafter until sacrifice. Control animals were inoculated with either drug or carrier protein alone in amounts equal to conjugated amounts. Blood was drawn on alternate weeks for determination of antidrug antibodies. Injections were continued until antibody titers dropped, which occurred at approximately 6 mo after initial injection. Antibodies directed against the immunogens were identified

Manuscript received October 24, 1978; accepted for publication April 3, 1979.

Melodie M. Buxman is the recipient of Clinical Investigator Award #AM 00391-01 from the National Institute of Arthritis, Metabolism and Digestive Diseases. This work was supported by grants from the National Institutes of Health, AM 17687-04, the Medical Research Foundation of Oregon and the Dermatology Foundation.

Presented in preliminary form at the annual meeting of the American Federation for Clinical Research, San Francisco, California, April 30, 1978 (Clin Res 26:297A, 1978).

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Abbreviations:

AIU: Amine-Incorporating Units
ENA: Extractable Nuclear Antigens
HSA: Human Serum Albumin
HYD: Hydralazine
INH: Isoniazid
RNP: Ribonucleoprotein
RSA: Rabbit Serum Albumin
TBS: 0.05 M Tris-0.15 M NaCl, pH 7.5

* 120 min was chosen as endpoint because epidermal transglutaminase approaches 50% initial activity at this time.

by Ouchterlony diffusion of experimental sera against the drug alone (using isoniazid crosslinked to casein), the immunizing drug-carrier complex, and the carrier protein alone.

Drug-specific antibodies to INH were quantitated in a radioimmunoassay conducted by modified Minden and Farr technique [10]. Aliquots of 0.1 ml of doubling dilutions of experimental serum were incubated overnight at 4°C with constant amounts (0.23 nM of specific activity 1.25×10^{-4} mC/mM) ^{3}H-INH bound to serum albumin (in a 0.1 ml volume). Following incubation, a 0.2 ml volume of saturated ammonium sulfate was added. Precipitates were centrifuged at 4°C and 3000 $\times g$ for 20 min, washed with cold 50% saturated ammonium sulfate, recentrifuged, and the radioactivity in the precipitate was counted in a well-type scintillation counter. Results were expressed as the ABC_{33} of the experimental serum. The ABC_{33} value represents the amount of ^{3}H-INH bound per ml undiluted serum at the antigen concentration employed (in this case, 0.23 nM of specific activity 1.25×10^{-4} mC/mM antigen). The ABC_{33} endpoint is the reciprocal of the serum dilution which precipitates 33% of the labeled antigen added.

RESULTS

Isoniazid was enzymatically crosslinked in linear fashion with time to rabbit serum proteins. Analysis of labeled serum by gel filtration chromatography showed that 60–80% of the recovered radioactivity eluted with a protein peak of 67,000 daltons in molecular weight which contained immunoreactive rabbit serum albumin by Ouchterlony diffusion. The remainder was distributed rather evenly among 3 other protein peaks which were not further identified. Purified RSA and HSA incorporated ^{3}H-INH similarly, 0.15 mole INH per mole albumin under identical experimental conditions, in 120 min of incubation time.

Histones coupled most rapidly to INH among the various nuclear constituents examined (Fig 1), and since they could be obtained in relatively purified form, were selected as a representative nuclear antigen for conjugation to INH and animal immunization. ENA incorporated lesser amounts of radioactivity and demonstrated a prolonged lag phase in accepting the drug. DNA and deoxyribonucleoprotein did not incorporate the drug. Specific activity of histone-INH conjugate was 0.02 mole INH per mole histone.

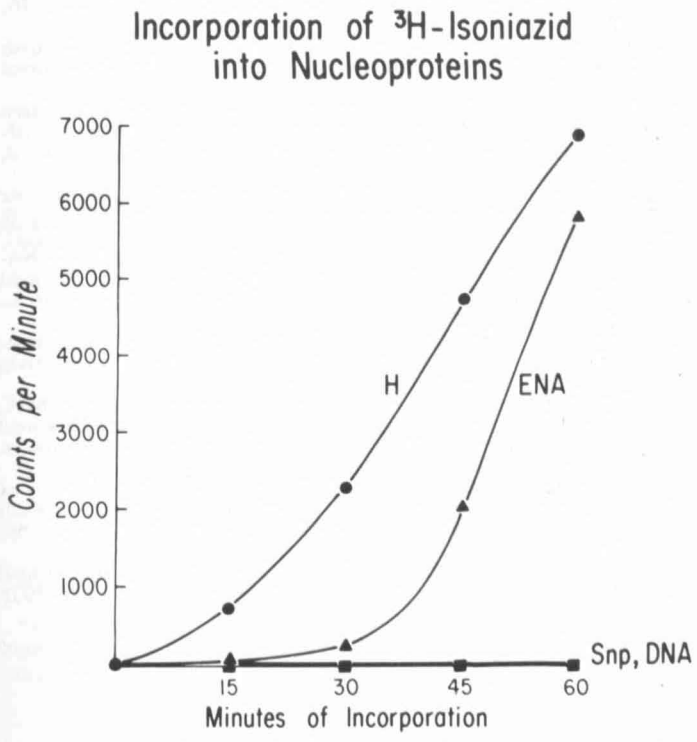

FIG 1. Incorporation of ^{3}H-INH into calf thymus nuclear proteins with time. *H* = histones; *ENA* = extractable nuclear antigens; *Snp* = deoxyribonucleoprotein; *DNA* = native DNA.

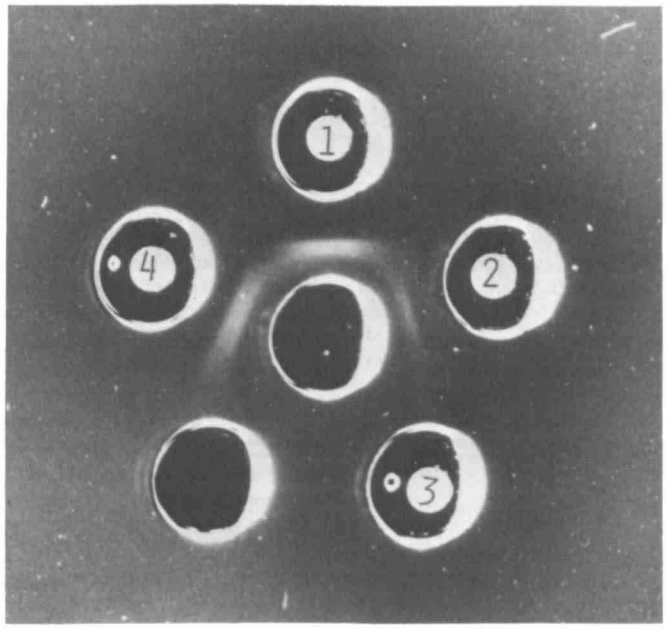

FIG 2. Ouchterlony plate showing INH-specific antibodies raised in rabbits immunized with INH crosslinked enzymatically to different proteins. *Center well* contains INH-casein; *Wells 1–4* contain, respectively, antisera directed against the following immunogens: INH-RSA, INH-HSA, INH alone, INH-histones.

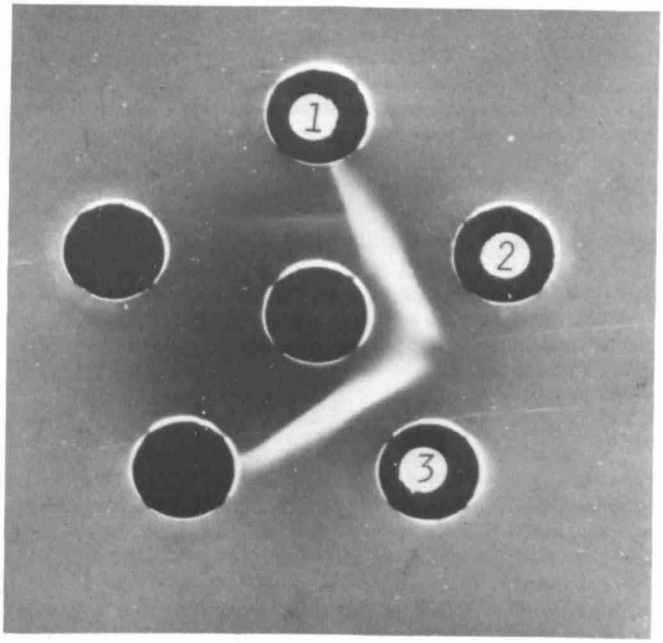

FIG 3. Ouchterlony plate demonstrating antibodies against both INH and heterologous carrier protein in rabbit immunized with INH-HSA. *Center well:* antiserum from immunized animal, diluted 1:1. *Well 1:* INH-casein. *Wells 2, 3:* human serum albumin.

In all animals receiving isoniazid crosslinked to a protein carrier, precipitating antibodies to the drug were detectable by the 4th week following primary immunization, independent of the carrier. At that time no animal had received more than the equivalent of 1 μg of drug antigen. The line of identity seen in the Ouchterlony plate in Fig 2, depicting sera from animals receiving different carriers diffused against INH-casein conjugate indicates that the INH determinant was antigenic in all animals. This precipitin line disappeared from Ouchterlony

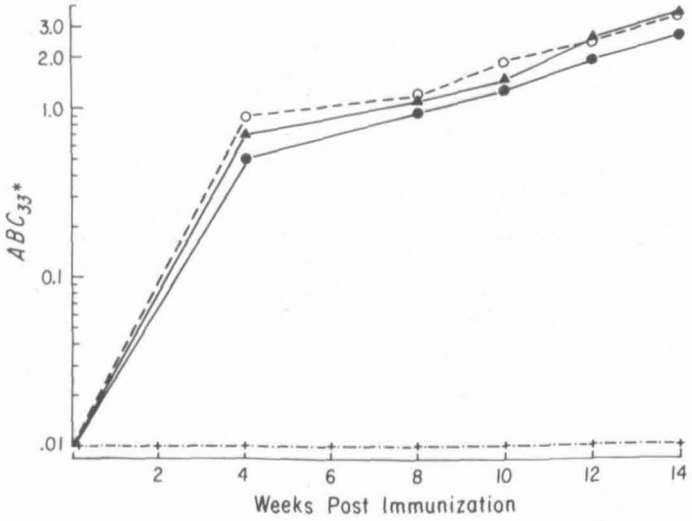

FIG 4. Antigen-binding capacity (ABC-33) of sera from rabbits immunized with INH crosslinked by transglutaminase to different carriers. Symbols: O---O, INH-histones; ▲——▲, INH-HSA; ●——●, INH-RSA; +.-.+, INH alone (see text for experimental details).

plates containing antisera previously absorbed with free isoniazid. Rabbits receiving only drug in adjuvant did not form antidrug antibodies; antibodies to carrier protein were observed only in animals injected with heterologous serum albumin (Fig 3).

Fig 4 demonstrates relative rises in drug antibody titer by radioimmunoassay. Antibody titers increased rapidly in all animals independent of protein carrier, and did not seem to be significantly elevated by the presence of the foreign HSA carrier. Again no antibodies were found in animals receiving unbound isoniazid. The minimum sensitivity of the radioimmunoassay was calculated at 5 ng bound conjugate.

Preliminary experience using hydralazine as primary amine substrate have yielded similar results. Hydralazine couples to RSA approximately 10 × more efficiently than INH under identical experimental conditions, suggesting that this drug may be a better substrate for transglutaminase. Drug-specific precipitin antibodies to hydralazine develop within 4 weeks of primary immunization.

DISCUSSION

The demonstration in this study that conjugation of a drug to a variety of cell nuclear proteins can be carried out with an intracellular transglutaminase offers a significant improvement over previous models for studying drug-induced hypersensitivity. Enzymatic coupling is gentle, and can be easily controlled by altering conditions of the reaction such as temperature, pH, enzyme concentration, or time. Selecting different carrier proteins, or increasing the rate and extent of drug crosslinking experimentally may produce conjugates capable of breaking tolerance to self-antigens.

It is tempting to speculate upon the relevance of this animal model to study of the pathogenesis of immune (e.g. vasculitis) and autoimmune (e.g. lupus-like) reactions to INH and HYD, as well as to other drugs such as procainamide. The incidence of drug-induced reactions to INH and HYD is time and dose-dependent [11,12]. Recent data suggest that a free amine group on the drug is essential for induction of drug hypersensitivity, since persons who are slow acetylators of the amine group of INH, HYD and procainamide have an increased incidence of immune reactions, and administration of N-acetyl procainamide prevents these reactions while retaining the drug's anti-arrhythmic properties [13]. Correlation of the presence of drug-specific antibody with symptomatic drug hypersensitivity is well documented, at least with respect to hydralazine [14]. INH induces a much lower incidence of hypersensitivity. One study (Yamauchi et al [15]) defined a definite relationship between antihydralazine antibodies and antinuclear activity using highly conjugated hydralazine-HSA conjugates. Antibodies to histone have recently been implicated in INH-induced hypersensitivity reactions [16].

Thus, the finding that transglutaminase-catalyzed drug-protein conjugates are highly immunogenic, and that such reactions are capable of occurring *in vivo* as well as *in vitro*, offers opportunity for more extensive investigations of controlled drug induced hypersensitivity in experimental animals and humans.

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