Pharmacology Biochemistry and Behavior, Vol. 58, No. 1, pp. 215–220, 1997

0091-3057/97 $17.00 + .00

ELSEVIER

PII S0091-3057(97)00005-1

Active Immunization with Cocaine–Protein Conjugate Attenuates Cocaine Effects

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Received 28 December 1995; Accepted 21 October 1996

ETTINGER, R. H., W. F. ETTINGER AND W. E. HARLESS. *Active immunization with cocaine–protein conjugate attenuates cocaine effects.* PHARMACOL BIOCHEM BEHAV **58**(1) 215–220, 1997.—Immunization with cocaine–keyhole limpet hemocyanin (KLH) conjugate elicited the formation of anti-cocaine antibody sufficient to blunt the effects of cocaine in rats. Cocaine was bound to KLH for immunization with the photoactivatable crosslinker *N*-hydroxysuccinimide-4-azidobezoate (HSAB). Immunization with the cocaine–KLH–complete Freund's adjuvant complex was effective in attenuating the analgesic and reinforcing effects of cocaine in laboratory rats. Enzyme-linked dot blot assay revealed the presence of anti-cocaine antibody in serum. Competitive binding studies suggest that the antibody was specific to cocaine. Active immunization for cocaine may provide an alternative to drug treatment and may provide protection from addiction. © 1997 Elsevier Science Inc.

Cocaine Antibodies Analgesia Reinforcement HSAB Rats

IT is estimated that 20–30 million people in the United States have used cocaine and that as many as 4 million people may be addicted. Illicit cocaine use has reached epidemic proportions, and there is no evidence that this trend is decreasing (10,12,21). Cocaine's powerful reinforcing effects are attributed to its ability to inhibit dopamine reuptake in the mesolimbic–cortical system. This inhibition agonizes dopamine neural transmission, resulting in reinforcement (9,19). Although the neural mechanisms of cocaine's effects are well understood, there appears to be no way to directly prevent them pharmacologically.

Recently, several laboratories have described cocaine-specific antibodies that may effectively interfere with cocaine's ability to agonize dopamine neural transmission. Catalytic antibodies, which promote rapid degradation of cocaine into its metabolites, ecgonine methyl ester and benzoic acid, have been isolated (14). Whether it is possible to develop catalytic antibodies powerful enough to block the reinforcing and analgesic effects of cocaine remains to be demonstrated. Haptens such as cocaine do not normally elicit active antibody formation. However, haptens may be conjugated with immunogenic proteins by a variety of cross-linking procedures [cf. (11)]. Immunization with cocaine conjugated with keyhole limpet hemocyanin (KLH) reportedly prompted the formation of anti-cocaine antibodies capable of preventing cocaine analgesia (2). This procedure required prior oxidation of cocaine ester bonds with sodium metaperiodate. The oxidized cocaine–KLH conjugate thus formed behaved as a stable immunogen. Although it is easy to visualize how preliminary metaperiodate oxidation can be used successfully in binding haptens containing vicinal hydroxyl groups to proteins, it is difficult to understand how such a process serves in coupling cocaine, which lacks vicinal hydroxyl groups, to KLH [cf. (1,8)]. Cocaine has also been coupled to KLH for immunization by structurally modifying the cocaine molecule (19). Immunization with this cocaine–KLH conjugate successfully elicited antibody formation sufficient to attenuate cocaine-induced locomotor activity and decrease brain levels of cocaine in immunized animals. We describe and evaluate an alternative coupling reaction for active immunization against the effects of cocaine.

It has been reported (7) that metoclopramide (MCP) was photolytically conjugated to bovine serum albumin (BSA) by using the crosslinker HSAB (*N*-hydroxysuccinimide-4-azidobenzoate). The bond formed was quite stable and the conjugate acted as a good immunogen. A free radical mechanism was postulated to account for the crosslinking process. Preliminary treatment of HSAB with BSA resulted in an amide derivative of the photoactive crosslinker by a free amino group in the BSA. Upon UV irradiation of the HSAB-derivatized BSA

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in the presence of MCP, free radicals were generated: a nitrene radical originating from the azido group, a chloride radical, and a phenyl radical resulting from homolytic cleavage of the C–Cl bond in the MCP aromatic ring. Among other products, interaction between the phenyl and nitrene radicals produced a diphenylamine derivative.

It occurred to us that in addition to the free radical crosslinking postulate, another process would be expected to predominate if the UV source was of slightly longer wavelength than that used to conjugate metoclopramide to BSA (7). In such a case, photolysis of azido groups may also give rise to nitrenes by loss of N_2. These reactive nitrenes have the propensity to form bonds of the electron donor–acceptor type. Because cocaine does not lack electron-donating groups, we decided to follow a procedure similar to that reported, using HSAB to crosslink cocaine to KLH and to BSA for immunization.

We examined the effects of immunization by evaluating cocaine's antinociception against thermal pain and reinforcement of place preference conditioning. Cocaine's antinociceptive effects are primarily mediated by blocking sodium conductance in peripheral pain neurons (20) and by dopamine mediation of projections to the medial thalamus (22), and cocaine's reinforcing effects are mediated centrally by dopamine agonism in the mesolimbic system (9,19).

METHODS

Preparation of Cocaine–KLH Immunogen

The cocaine–KLH conjugate was prepared by first dissolving HSAB (4 mg) in 100 μl of dry DMSO; then 12 μl of this solution was added to a solution of KLH (10 mg in 1 ml of 50 mM phosphate buffer, pH 7.4). The HSAB was allowed to react with the KLH at room temperature in the dark for 10 min, after which excess HSAB and DMSO were removed by gel filtration through a column of Sephadex-G10 (0.8 × 16 cm) equilibrated in phosphate buffer. The KLH-containing fractions (6 × 0.5 ml, determined by absorption of UV light at 280 nm) were pooled, chilled on ice, and then mixed with 68 mg cocaine HCl. Photoactivation of the HSAB–KLH conjugation in the presence of cocaine was performed in a 3-ml quartz cuvette placed in an ice bath 9 cm from a 450-W immersion-type UV photochemical lamp (Ace Glass). Photoactivation was allowed to proceed for 5 min. The cocaine–KLH conjugate was separated from nonconjugated cocaine by exhaustive dialysis against 150 mM NaCl, 50 mM phosphate buffer, pH 7.4 (PBS). A cocaine–BSA conjugate and a [^{3}H]cocaine–KLH conjugate were also prepared by the same method.

The amount of cocaine conjugated to KLH by HSAB was determined by performing the coupling reaction with [^{3}H]cocaine (14.1 GBq/mmol). Samples of the reaction were spotted onto glass fiber filters (Fisher G4) before and after photoactivation. The filters were allowed to dry and were subsequestly assayed for trichloracetic acid (TCA) precipitable radioactivity (16).

All chemicals, except [^{3}H]cocaine, were obtained from Sigma Chemical Company (St. Louis, MO, USA). [^{3}H]Cocaine was obtained from New England Nuclear (Wilmington, DE, USA).

Animals

Thirty-six experimentally naive female Long–Evans rats (Charles River, Wilmington, MA, USA) 9 months of age were used. Animals were randomly divided into three groups of 12 for immunization. All animals were housed individually in suspended stainless steel cages (17.75 × 24.5 × 17.75 cm) in a climatically controlled room (22°C) with 24-h illumination. Food and water were available ad lib.

Immunization with Cocaine–KLH Conjugate

Animals in the immunization group (n = 12) were injected with 0.2 ml cocaine–KLH emulsified with 0.2 ml complete Freund's adjuvant (CFA). A second group of 12 animals was injected with 0.2 ml of the KLH–HSAB compound photoactivated in the absence of cocaine and emulsified with 0.2 ml CFA (KLH–HSAB control). A third group of 12 animals was injected with 0.2 ml KLH in saline emulsified with 0.2 ml CFA (KLH control). Four weeks after the initial injections, and at 4-week intervals throughout, all animals received booster injections with the same immunogen emulsified with incomplete Freund's adjuvant. All immunizations were administered subcutaneously to the anterior dorsal area of the back.

Hot-Plate Reaction Test

We first examined the effectiveness of the cocaine–KLH conjugate in blocking cocaine's effects by measuring reactions of immunized animals to a hot plate following cocaine administration. Cocaine exerts antinociceptive effects both peripherally by blocking sodium conductance (20) and centrally via dopamine projections to the medial thalamus (22). A Thermolyne hot plate (model HPA1915B, Barnstead/Thermolyne Corporation) was modified by addition of a 16.8 × 11.1 × 0.4-cm steel plate for increased temperature regulation, a four-walled Plexiglas cage (16.8 × 11.1 × 30.5 cm), and a composite wood lid (25.4 × 25.4 × 0.4 cm) to confine animals to the hot plate. A remote thermometer was used to monitor the surface temperature of the hot plate.

Three separate hot-plate tests were administered to the cocaine–KLH-immunized and KLH control animals over a 3-week period. Hot-plate reaction times following either saline or cocaine administered intraperitoneally (IP) were recorded on alternate days for each test. Five weeks postimmunization (1 week after booster injections), all subjects were administered 0.25 ml 9% saline solution IP, returned to their home cage for 15 min, and then tested on the hot plate (54°C). The following day, all animals were injected IP with 25 mg/kg cocaine HCl, returned to their home cage, and tested on the hot plate 15 min later. The order of injections was reversed for the second hot-plate test conducted 6 weeks postimmunization, and reversed again for the third hot-plate test conducted 7 weeks postimmunization. The hot plate was maintained at 54°C for all subjects during all trials. Animals remained on the hot plate until paw licking or a limb withdrawal reflex occurred, or until 45 s had elapsed. No animals were allowed to stay on the hot plate for more than 45 s, and no animals suffered burns or severe discomfort.

Place Preference Conditioning

The place preference conditioning (PPC) method has been used to reliably evaluate the reinforcing properties of drugs, including cocaine, because it allows for a rapid and direct assessment of a learned association between a drug and specific environmental stimuli (17,18,23). We used cocaine-reinforced PPC to evaluate the reinforcing properties of cocaine following immunization. Conditioning trials were conducted in a wooden rectangular chamber using the same 24 animals from the cocaine–KLH immunization and KLH control groups. The chamber was divided into three separate rooms separated by guillotine doors. The two end compartments measured 31 × 27 ×

25 cm high. The middle compartment was 20 × 10 × 25 cm high. One end compartment was painted dark green, had a black top, and was not illuminated. The other end compartment was white, had a clear Plexiglas top, and was illuminated with a 25-W light located 0.5 m above the chamber. Both end compartments had wire mesh floors and were suspended above cedar shavings. The center compartment was grey and had a clear Plexiglas top and a metal floor. A mirror located above the center compartment allowed an observer to record when animals entered or left either end compartment. White noise masked extraneous sounds.

Place preference conditioning commenced 8 weeks post-immunization. Phase 1 consisted of four consecutive days of baseline training followed by a place preference test. During training, animals were placed in the center chamber of the PPC apparatus with the guillotine doors closed; after 1 min the doors to both end compartments were opened and the animals were allowed to explore the chamber unrestricted. After 15 min the animals were removed and returned to their home cage. On the fifth day, time spent in each compartment was recorded during a 15-min test. An animal was considered to be in an end compartment only when its two front paws were in that compartment. Phase 2 consisted of eight consecutive days of cocaine-reinforced PPC followed by another place preference test. During conditioning, each animal was administered either 0.25 ml 9% saline or 10 mg/kg cocaine HCl in saline IP on alternate days. After saline administration, the animals were restricted to the dark compartment for 15 min, then returned to their home cage. Following cocaine administration, the animals were restricted to the white, lighted compartment for 15 min, then returned to their home cage. On the ninth day, the animals were not injected and were placed in the central compartment with the doors to both end compartments open for 15 min. The time spent in each compartment was recorded as before. Phase 3 consisted of 8 days of conditioning in which the PPC of phase 2 was reversed. All of the procedures in phase 2 were followed except that animals were placed in the white, lighted compartment immediately following saline injections and in the dark compartment following cocaine injections. The animals were again tested on the ninth day, using the same procedure as in phase 2.

RESULTS

Immunoassay for Anti-Cocaine Antibody

Immunoassays for cocaine antibodies were performed on blood drawn from tail veins of all 36 animals 13 weeks after initial immunizations (1 week after the third booster injections). Approximately 0.25 ml of serum from each animal was frozen until assays were performed over the following 2 weeks. Enzyme-linked dot blot assays for anti-cocaine antibodies were performed for each serum sample. First, serial dilutions (in PBS) of the cocaine–KLH immunogen, cocaine–

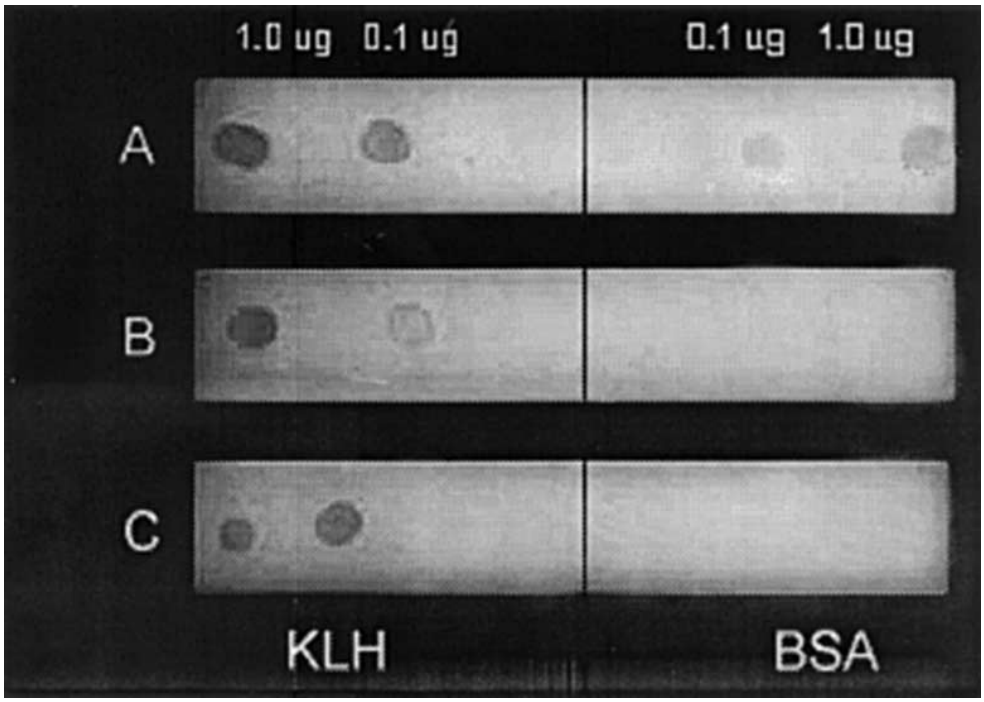

FIG. 1. Representative dot blot assays for anti-cocaine antibody. Two dilutions (1.0 μg and 0.1 μg) of cocaine–KLH were placed on the left side of strips A–C. Two dilutions of cocaine–BSA (1.0 μg and 0.1 μg) were placed on the right side of strips A and C. The right side of strip B contained two dilutions (1.0 μg and 0.1 μg) of BSA alone. Strips A and B were incubated with serum from a representative animal immunized with cocaine–KLH. Binding of antibodies to the strips is indicated by staining. Binding of antibody to cocaine–KLH (A and B, left side) and cocaine–BSA (right side of A, indicated by arrows), but not to BSA alone (B, right side), indicates the presence of anti-cocaine antibody in this animal. Strip C was incubated with serum from a representative animal immunized with KLH alone. Binding of antibodies to cocaine–KLH (C, left) indicates the presence of anti-KLH antibody. The absence of binding to cocaine–BSA (C, right) indicates the lack of anti-BSA or anti-cocaine antibody in this animal.

BSA conjugate, and BSA alone were bound to nitrocellulose. After drying, the nitrocellulose was blocked with nonfat milk–Tris buffered saline solution and then incubated with 0.25 ml serum in 25 ml nonfat milk for 2 h (13). The nitrocellulose was washed extensively in nonfat milk and then incubated for another 2 h with alkaline phosphatase-conjugated goat anti-rat IgG (Sigma) diluted 1:2,000 in nonfat milk. Enzyme-linked anti-rat IgG was detected by using X-phos and nitroblue tetrazolium (3). Serum from 11 of the 12 animals immunized with KLH–cocaine conjugate revealed cocaine antibodies. Anti-cocaine antibody was not detected by this method in any of the KLH control or the KLH–HSAB control animals. Representative nitrocellulose strips are presented in Fig. 1. The left half of each strip shows antibody binding to dilutions of cocaine–KLH. The presence of anti-cocaine antibody is demonstrated by the anti-rat IgG binding to cocaine–BSA on the right half of the strip in Fig. 1A. There was no evidence of anti-rat IgG binding to the right half of the strip in Fig. 1B, which contained BSA alone. The strip in Fig. 1C shows KLH antibody binding (left half) and no cocaine–BSA binding (right half) for KLH control animals. Animals immunized with KLH–HSAB photoactivated in the absence of cocaine also revealed KLH antibody binding and no cocaine–BSA binding (not shown).

To determine the specificity of the anti-cocaine antibody, 0.25 ml of serum from cocaine–KLH-immunized animals was incubated for 20 min in 25 ml nonfat milk in the presence of 25 mg free cocaine before incubating with nitrocellulose. None of these samples revealed anti-rat IgG binding to cocaine–BSA (not shown), indicating that the antibody present was specific to cocaine.

Hot-Plate Reaction Test

Mean differences (D in Table 1) in hot-plate reaction times for the saline vs. 25-mg/kg cocaine injections are compared in Fig. 2 within groups for all three hot-plate tests. Statistical comparisons were made with paired *t*-tests and are presented in Table 1. For each test, there were no differences in hot-plate reaction times following saline and cocaine administration for cocaine–KLH-immunized animals. All three tests revealed significant differences, however, in reaction times following saline and cocaine administration for the KLH control animals. These significant increases in hot-plate reaction times for the control animals suggest that cocaine attenuated thermal pain in these animals but not in the immunized animals. These results indicate that immunization with cocaine conjugates can successfully blunt cocaine analgesia and that this immunization effect is not transient.

Place Preference Conditioning

The results of cocaine-reinforced PPC are presented in Fig. 3. The figure shows mean proportions of test time spent in the white, lighted area during each phase of conditioning. There were significant differences in place preference for the KLH control animals but not for the cocaine–KLH-immunized animals across preference tests ($F = 6.47$, $p = 0.008$). That is, the KLH control animals demonstrated PPC and PPC reversal using cocaine as a reinforcer. On the other hand, the cocaine–KLH-immunized animals failed to demonstrate conditioning with cocaine during either phase 2 or 3. Because the mean baseline preference for the white area was slightly higher for the cocaine–KLH animals (0.55 vs. 0.43 for the KLH control animals, $t = 1.05$, $p = 0.32$), the failure to demonstrate PPC for the immunized animals in phase 2 might be attributed to a ceiling effect. Therefore, we reversed PPC during phase 3. Even though the preferences for the white area following phase 2 were identical for both groups (0.63 vs. 0.63), only animals in the KLH control group showed a reversal in their white area preference following conditioning with cocaine to the black area in phase 3 ($t = 2.85$, $p < 0.02$). These results demonstrate that active immunization with cocaine conjugate blunts cocaine's reinforcing effects as measured by PPC.

TABLE 1

STATISTICAL COMPARISONS OF THE MEAN DIFFERENCES (D) IN HOT-PLATE REACTION TIMES FOLLOWING SALINE AND COCAINE ADMINISTRATIONS FOR CONTROL AND COCAINE-IMMUNIZED ANIMALS

Group	Test	D	*t*	df	*p*
Control	1	8.50	2.15	11	0.027
	2	17.77	2.35	11	0.019
	3	14.09	4.66	11	0.000
Immunized	1	0.50	0.00	10	1.00
	2	3.33	1.08	10	0.300
	3	0.77	0.55	10	0.590

Results for each of the three hot-plate tests are shown. Cocaine significantly increased hot-plate reaction times in all three tests for the control animals but not for the immunized animals.

DISCUSSION

Animals immunized with the cocaine–KLH–CFA conjugate demonstrated resistance to both cocaine's thermal analgesic and reinforcing effects. Cocaine analgesia to thermal pain was evaluated by measuring reaction times of animals placed on a standard hot plate. Cocaine reinforcement was evaluated by place preference conditioning. Because antinociception for thermal pain is primarily mediated by peripheral neurons and cocaine reinforcement is primarily mediated by central dopamine neurons, our results suggest that anti-cocaine antibody prevents cocaine effects in both peripheral

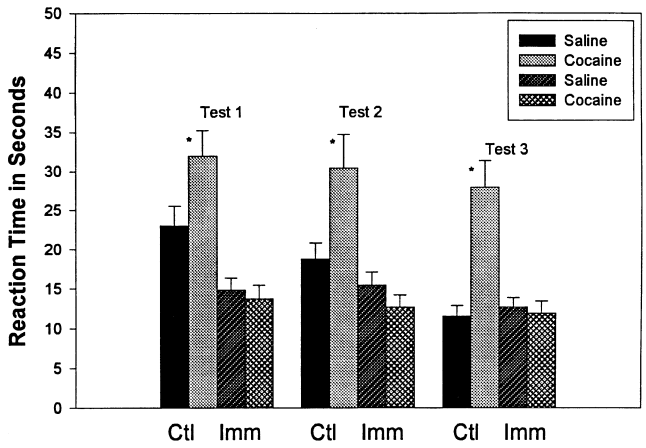

FIG. 2. Mean hot-plate reaction times for three separate hot-plate tests. Each test (1–3) shows the mean reaction time for control (Ctl) and immunized (Imm) animals subsequently treated with saline or 25 mg/kg cocaine, respectively. Cocaine significantly increased hot-plate reaction times for the control animals only in all three tests. Asterisks indicate significant differences from the prior condition.

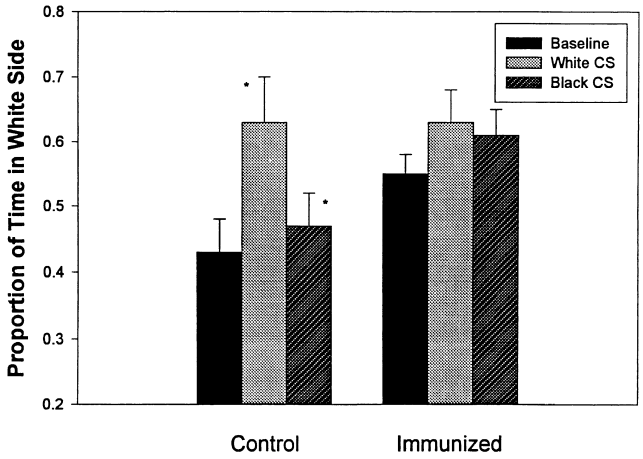

FIG. 3. Mean proportions of time in the white side following baseline exposure (Baseline), cocaine-reinforced PPC to the white area (White CS), and cocaine-reinforced PPC to the black area (Black CS) for KLH control and cocaine–KLH-immunized animals. Control animals showed increases in preference for the white area following conditioning to white and increases in preference for the black area following conditioning to black. Immunized animals did not show changes in place preference. Asterisks indicate significant differences from the prior condition.

and central neurons. Furthermore, the anti-cocaine antibody elicited in our animals did not appear to undergo depletion with relatively large cocaine doses (25 mg/kg in our hot-plate tests and 10 mg/kg in our PPC tests) or with repetitive cocaine administration. We observed strong cocaine effects in our control animals throughout testing, indicating that animals were not developing tolerance to repeated cocaine exposure. These results both support and extend those reported previously (2,4,5,15). Although we have not yet determined the level of anti-cocaine antibody present in the immunized animals, the anti-cocaine antibody appeared to be specific to cocaine, because competitive binding with free cocaine in serum prevented binding to our cocaine–BSA conjugate.

Cocaine is sensitive to enzymatic hydrolysis of the methyl ester resulting in the production of benzoylecgonine (4,6). It has been suggested that conjugates designed to elicit anti-cocaine antibody production that retain the methyl ester may become deesterified in vivo, resulting in an appreciable anti-benzoylecgonine titre, which is expected to interfere with production of optimal levels of anti-cocaine antibodies (4). The conjugate prepared in the current report should be as sensitive to methyl ester hydrolysis as is free cocaine under physiological conditions. However, because the methyl ester represents only a single epitope of the entire cocaine molecule, the deesterified conjugate should still elicit an antigenic response, resulting in production of polyclonal antibodies, many of which may still recognize cocaine by its other functional groups. The conjugation method proposed here, using HSAB, likely results in several different orientations of the exposed cocaine molecule, and a broad spectrum of anti-cocaine antibodies may have been elicited. Therefore, it may never be clear what proportion of the antibody population was elicited by the conjugated metabolites ecgonine methyl ester or benzoylecgonine. Nevertheless, the HSAB conjugation method is rapid and relatively efficient and results in a product that elicits the production of anti-cocaine antibodies. An estimate of the efficiency of binding cocaine to KLH by this method was obtained by conjugating [^{3}H]cocaine to KLH. This analysis revealed that 60% of the theoretical number of HSAB molecules on KLH were conjugated to cocaine. Whether other conjugation methods yield a product that more efficiently elicits the production of anti-cocaine antibodies has yet to be determined.

Further behavioral and immunological studies are currently in progress to quantify the amount of cocaine antibody present in rat serum and to explore the long-term effectiveness of cocaine antibody in attenuating the drug's reinforcing properties and drug-induced analgesia. Active immunization against cocaine using conjugation methods may provide an adjuvant to drug treatment and provide protection from addiction.

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