NICOTINE ANTIBODY PRODUCTION: COMPARISON OF TWO NICOTINE CONJUGATES IN DIFFERENT ANIMAL SPECIES

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Received September 22,1975

Summary. Preparation and characterization of two nicotine-albumin conjugates, N-Succinyl-6-amino-DL-nicotine-BSA and 6-(Σ-aminocapramido)-DL-nicotine-BSA are described. Ten and eleven molecules of nicotine per molecule of BSA were found, respectively. Two different animal species were used to produce anti-nicotine antibodies. Goats inoculated with 6-(Σ-aminocapramido)-DL-amino nicotine conjugate produced antibodies with higher titer and better affinity and specificity. Comparative studies of antibody production, purification, solid phase assays in controlled pore glass, titration and specificity are also reported.

Introduction. Conventional assay methods for the detection of nicotine in biological fluids are laborious, time-consuming and not very sensitive. Radioimmunoassays have been developed for many low molecular weight substances by our laboratory and others (1-7). Recent reports indicate that antibody developed by conjugation of nicotine to protein carriers has been of low titer, sensitivity and specificity (8,9). The present investigation was initiated to produce large quantities of nicotine antibodies in different animal species suitable for development of a solid-phase radioimmunoassay. In this type of assay, high titer and specificity are germain.

The low antigenicity, lack of a functional group, and the need for preservation of the antigenic pyrrolidine ring moiety in nicotine requires the introduction of a functional group and a fairly long rigid linkage to the macromolecular carrier. This paper deals with the preparation of two nicotine protein conjugates, the specific characterization of one of these conjugates, and the production of antibodies in two different animal species.

Methods

A. N-Succinyl-6-Amino-DL-Nicotine-BSA Conjugate

6-amino-DL-nicotine was prepared and isolated according to the method of Tschitschibabin (10). ir (net) 3460, 3330, 3195 (m, NH), and 1625 cm^{-1} (s, NH); nmr ($CDCl_3$). δ 2.13 (S (superimposed on a broad CH) 3H), 1.45-2.40 (m, 5H), 2.70-3.00 (mt, 1H), 3.05-3.30 (mt, 1H), 5.06 (S, 2H), 6.46 (d, $J_{5,4}$ = 8.2 cps, 1H), 7.42 (dd, $J_{4,5}$ = 8.2 cps, $J_{4,2}$ = 2.2 cps, 1H), and 7.90 d, $J_{2,4}$ = 2.2 cps, 1H). The 6-amino-DL-nicotine was succinylated with succinic anhydride.

Conjugation of N-succinyl-6-amino-DL-nicotine to BSA protein carrier was carried out by carbodiimide condensation at pH 5.5 in distilled water. After one hour, 30 mg of water soluble carbodiimide and 30 mg of succinyl nicotine were added in addition to the starting amount to ensure sufficient conjugation and dialized for three days in 10 liters of phosphate buffer (0.01M; pH 7.8). The nicotine/BSA ratio was verified by ultraviolet spectroscopy and found to be 10. N-succinyl-6-amino-DL-nicotine in presence of water soluble carbodiimides will undergo internal cyclization. The conjugate was found to be labile and was used as soon as possible after conjugation was complete.

B. 6-(Σ-Aminocapramido)-DL-Nicotine-BSA Conjugate

6-amino-DL-nicotine was prepared as described above.

Σ-N-t-Butyloxycarbonyl) caproic acid was prepared according to the method of Schwyzer (11). A mixture of 13.18 gm (0.1 mole) of ε-aminocaproic acid, 22 gm (0.15 mole) of t-butyloxycarbonyl azide and 8 gm (0.2 mole) of magnesium oxide in 120 ml of water/200 ml of diozane was stirred for 20 hours between 40-45°C. The reaction mixture was cooled and the magnesium oxide was removed by filtration. The filtrate was washed three times with ether to remove unreacted azide. The aqueous phase was chilled in ice, acidified with solid citric acid to pH 3.5, and then extracted three times with ethyl acetate. The organic phase was washed three times with saturated sodium chloride solution, dried over anhydrous sodium sulfate and filtered. A clear oil was obtained upon removal of the solvent. Crystallization from pet-ether (bp. 30-70°C) yielded 19.1 gm (8.26% yield) of white solid mp 35.5-37°C. Electrophoresis (HCOOH/CH_3COOH/H_2O:6/13/100, pH 5.0) showed only one spot with tolidine/chlorine reagent and was ninhydrin negative. TLC (silica gel) ethyl acetate; methanol/chloroform (1:1), and isopropanol/1M ammonium hydroxide (2:1) systems gave only one spot with tolidine/chlorine reagent and was ninhydrin negative. ir ($CHCl_3$) 1167 (s, C - O), 1698, 1704, 1712 (s, C - O), and 3460 cm^{-1} (m, NH), nmr ($CDCl_3$), 1.43 (S (superimposed on a broad CH), 9H), 1.20-1.80 (m, 6H), 2.34 (t, J = 7 cps, 2H), 3.07 (broad t, J = 6 cps, 2H), NH at 5.00 and 6.25 (1H) and the OH at 11.24 (1H). The dicyclohexylamine salt was prepared, recrystallization from ether and petroleum ether (bp. 30-70°C) gave white crystals mp 93.5-94.5° C.

Anal. calculated for $C_{23}H_{44}N_2O_4$:C, 66.95; H, 10.75; N, 6.79.

Found: C, 66.21; H, 11.05; N, 6.74.

6-(ε-aminocapramido)-DL-nicotine. Isobutylchloroformate (1.32 ml; 10 mmole) was added slowly to a cooled solution of 2.313 gm (10 mmole) of ε-BocCaproic acid and 1.4 ml (10 mmole) of triethylamine in 20 ml of dry toluene at -5°C. After 25 minutes at this temperature, 1.25 mmole of 6-amino-DL-nicotine in 5 ml of dry toluene was added. Rapid CO_2 evolution began immediately; the reaction proceeded overnight at room temperature. Triethylamine hydrochloride was removed by filtration and the toluene was removed under reduced pressure. Ethyl acetate was added to the yellowish oily product; which was washed four times with a solution of 10% sodium bicarbonate saturated in sodium chloride, three times with distilled water, dried over anhydrous sodium sulfate and filtered. The solvent was removed under reduced pressure and the gum-like product was dissolved in ether, dried over anhydrous sodium sulfate, and yielded 1.81 gm (46% yield) of oily product upon removal of the solvent. Electrophoresis ($CH_3COOH/C_6H_5N/H_2O$ 22.8/32.3/41, pH5) indicated a trace of 6-amino-DL-nicotine in the product. ir (CCl_4) 1170 (s, C-0), 1698, 1704, 1724, (s, C-O). 3220, 3435 and 3465 cm^{-1}(m, NH). The 6-(ε-aminocapramido)-DL-nicotine was obtained by removal of the Boc-group from 6-(ε-BocCapramido)-DL-nicotine using hydrogen chloride in tetrahydrofuran.

6-(ε-aminocapramido)-DL-nicotine-BSA conjugate. A solution of 192 mg of BSA in 3 ml of water was added to a solution of 847 mg of 6-(ε-aminocapramido)-DL-nicotine in 10 ml of water which was previously adjusted to pH 5 at room temperature. 1-ethyl-(3.8-dimethylaminopropyl)carbodiimide hydrochloride (650 mg) was added in one portion to the above solution with stirring. The pH of the reaction was maintained at 5 through the first two hours. An additional 650 mg of carbodiimide was added after one hour and the reaction mixture was allowed to stand at room temperature for 72 hours. The conjugate was dialized for three days in 8 liters of phosphate buffer (0.01M, 7.8 pH) and finally one day in sodium chloride/phosphate buffer (0.01M, 7.8 pH) (The buffer solution was changed twice daily). It was found that the nicotine/BSA ratio was 11 based on tracer method calculations.

Administration of immunogens. A saline suspension of antigen emulsified with an equal volume of complete Freund's adjuvant was injected into 6-lb (average) New Zealand rabbits (R), and into 50-lb (average) goats (G). The schedule of injection in the different groups was as follows:

Group (I) (N =4) Rabbits. N-succinyl-6-amino-DL-nicotine-BSA, 2 mg of conjugate/rabbit in 1 ml, in toe pads of all four feet (0.1 ml/site); 2 weeks later each rabbit was injected in both hind legs with 0.5 ml of the emulsion (2 mg conjugate in .5 ml saline and .5 ml of complete Freund's adjuvant). Two weeks later the dose was repeated in both hind legs and harvesting of antibodies was started one week after the third injection.

Group (II) (N =3) Goats. N-succinyl-6-amino-DL-nicotine-BSA, 1 mg of conjugate/goat. The conjugate suspension was emulsified with complete Freund's adjuvant as described above, and 0.25 ml of the emulsion was injected intramuscularly into the proximal part of each leg. The goats were immunized at monthly intervals for three months, and then every two months thereafter. Harvesting began one week after the third primary injection.

Group (III) (N =4) Rabbits. 6-(ε-aminocapramido)-DL-nicotine-BSA conjugate was prepared and injected in the same amounts and schedule as described for Group I for N-succinyl-6-amino-DL-nicotine conjugate.

Group (IV) (N =3) Goats. 6-(ε-aminocapramido)-DL-nicotine-BSA conjugate was also prepared and injected in the same amounts and schedule as described for Group II.

Immobilization of Antisera. The solid support (controlled pore glass) was obtained from Corning Glass Works. Diazotization of the glass arylamine groups was previously reported by our laboratory (12). The N-Hydroxy Succinimide controlled pore glass was obtained from Pierce Co. Ten ml of 0.1 M phosphate buffer containing 10 mg/ml of anti-nicotine (pH 7.4) and 1 gm of activated support material at 4°C was gently agitated and evacuated for two hours to degas and to insure liquid contact throughout the control glass pores. Coupling was terminated by reaction of 1.0 M glycine. The complex was stored in a closed plastic container at 4°C.

Results and Discussion

Antibody Response. None of the rabbits of Groups I and III (N-succinyl-6-amino-DL-nicotine-BSA and 6-(ε-aminocapramido)-DL-nicotine-BSA produced a useful antisera. Titers were very low.

Groups II and IV (goats) produced antibodies which had sufficiently high titers to be useful in routine nicotine determinations.

Groups I and III inoculated with N-succinyl-6-amino-DL-nicotine-BSA conjugate produced titers in the range of 1:25 to 1:50. Titer was not increased with monthly booster shots (Fig. 1).

Group IV, inoculated with 6-(ε-aminocapramido)-DL-nicotine-BSA conjugate produced higher titers (1:100 to 1:200) than any of the groups of animals. Antiserum from group IV was used for routine nicotine assays. The lowest detectable quantity of nicotine was approximately 1 ng (Fig. 2).

In general, reliable prediction of maximal antibody response

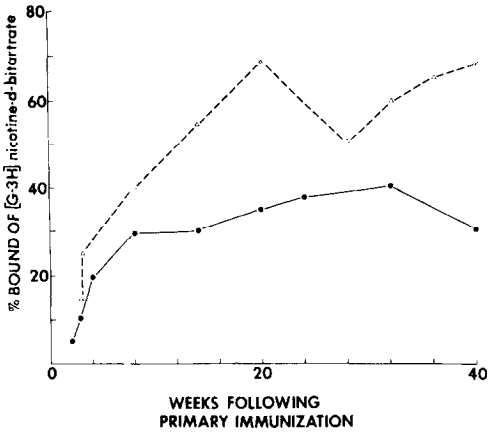

Fig. 1. Antibody Production (average of Group (II) Goats, immunized with N-Succinyl-6-amino-DL-nicotine-BSA ●——● and Group (IV) Goats, immunized with 6-(ε-aminocapramido)-DL-nicotine-BSA Δ----Δ; antiserum with higher titer and affinity was obtained.

Fractionation of Serum. Fractionation of goat antiserum was done previous to immobilization on inorganic support as follows: Goat antisera was fractionated with $(NH_4)_2 SO_4$, recovered by centrifugation, and then resuspended in 0.5 vol. of normal saline.

The solution was dialized against 10 liters of phosphate buffered saline (5mM sodium phosphate, pH 7.5, containing 150 mM NaCl). Dialysis was continued for 72 hours with two buffer changes.

in animals requires much experimentation with a large number of animals, many inbred strains and assessment of such variables as carrier molecules, linkages, sites of injection, dose of immunogen, intervals between boosters, etc. It is common knowledge that species and individuals within a species vary in response to antigen after injection of a given antigen conjugate; some producing high titer antibodies and others exhibiting little or no response.

Solid phase radioimmunoassay of nicotine was found to be non-practical since the titer of anti-nicotine was low. During displacement studies with immobilized anti-nicotine antibodies

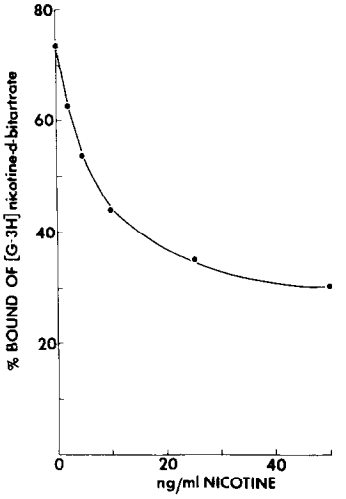

Fig. 2. Displacement of binding of (G-^{3}H) nicotine-d-bitartrate by non-radioactive nicotine. The affinity constant was calculated to be 2.1 x 10^{-7}M.

Antisera titer. The titer of each antisera was assessed by measuring the ability of antisera to bind labeled nicotine. Dilutions of 1:10, 1:25, 1:50, 1:100, 1:200, 1:500 of each antisera was incubated with a previous dry amount of 3000 cpm of (G-^{3}H) nicotine-d-bitartrate. Antisera was diluted in 0.5 ml of 0.05 m phosphate buffer, pH 8.1 containing 0.1% BSA. After 14 hours of incubation at 4°C, separation was carried out by a solution of dextran-coated charcoal containing 0.1% of Norit A RIA grade charcoal and 0.1% of dextran T-70. Tubes were well mixed and let stand in a 4°C water bath for 10 minutes, and then centrifuged for 20 minutes at 2,500 rpm. Supernatant was counted in an Isocap liquid scintillation spectrometer.

the curves obtained were almost flat. Immobilized antibodies in inorganic material were found to be unstable and of short shelf life.

Further studies are in progress in our laboratory to produce specific L-nicotine compounds that can be used as antigens to produce anti-nicotine antibodies and also be capable of labelling with high specific activity Na^{125}I, which will increase many times the sensitivity of radioimmunoassay of nicotine.

Acknowledgement. We wish to thank Mr. Alfred Chung for invaluable help with the synthesis of these compounds. This research was supported by Grant 884M from the Council for Tobacco Research USA, Inc. and the Florida Juvenile Diabetes Research Foundation.

References

1. Castro, A., Shi, T. and Chung, A. (1973) Experientia 29: 1447-1448.
2. Castro, A., Jelen, B. and Kutas, M. (1973) Experientia 29: 1581-1582.
3. Castro, A., Shi, T. and Chung, A. (1974) Steroids 23: (5) 625-638.
4. Cheng, L.T., Kin, S.Y., Chung, A. and Castro, A. (1973) FEBS Letters 36: 339-342.
5. Castro, A., Grettie, D., Bartos, D. and Bartos, F. (1973) Res. Commun. in Path and Pharm. 6: 879-886.
6. Chung, A., Kin, S.Y., Cheng, L.T. and Castro, A. (1973) Experientia 29: 820-821.
7. Spector, S. and Parker, C.W. (1970) Science 168: 1347-1348.
8. Matsushita, H., Nogudii, M. and Tamaki, E. (1974) Biochem. Biophys. Res. Commun. 57: 1006-1010.
9. Langone, J.L., Gjika, H.B. and VanVunakis, H. (1973) Biochemistry 12: 5025-]029.
10. Tschitschibabin, H.E. and Kirssanow, A.W. (1924) Chen. Ber 57: 1163-1168.
11. Schwyzer, R., Sieber, P. and Kappeler, H. (1959) Helv. Chim. Act. 42: 2622-2624.
12. Castro, A., Ugarte, E. (1974) Res. Comm. in Chem. Path and Pharma. 7: 455-458.