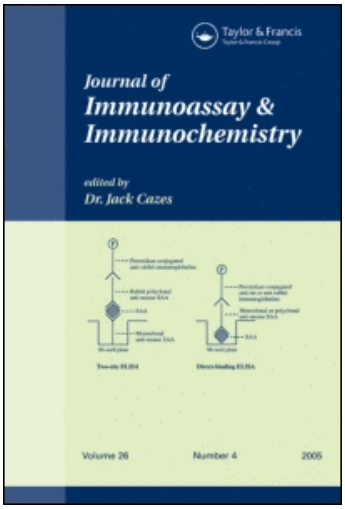

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Antiserum for Insulin Radioimmunoassay Generated by an Insulin Derivative Devoid of Hypoglycemic Activity

Ibrahim F. Heneine[a]; Paulo Salgado[b]; Maria C. S. Nascimento[a]; Mercia P. Lima[a]
[a] Biophysics Laboratory. Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais, Belo Horizonte, MG [b] BIOBRÁS S.A., Montes Claros, MG, Brazil

To cite this Article Heneine, Ibrahim F. , Salgado, Paulo , Nascimento, Maria C. S. and Lima, Mercia P.(1993) 'Antiserum for Insulin Radioimmunoassay Generated by an Insulin Derivative Devoid of Hypoglycemic Activity', Journal of Immunoassay and Immunochemistry, 14: 1, 51 — 61
To link to this Article: DOI: 10.1080/15321819308019840
URL: http://dx.doi.org/10.1080/15321819308019840

JOURNAL OF IMMUNOASSAY, 14(1&2), 51-61 (1993)

ANTISERUM FOR INSULIN RADIOIMMUNOASSAY GENERATED BY AN INSULIN DERIVATIVE DEVOID OF HYPOGLYCEMIC ACTIVITY.

Ibrahim F. Heneine[1*], Paulo Salgado[2],
Maria C.S. Nascimento[1], and Mercia P. Lima[1].

Biophysics Laboratory. Department of Physiology and Biophysics. Institute of Biological Sciences, Federal University of Minas Gerais, 31 270-901, Belo Horizonte, MG, and [2]BIOBRÁS S.A., 39 400-001, Montes Claros, MG Brazil.

ABSTRACT

A tetraiodinated derivative of bovine insulin, prepared at pH 1 with stable iodine, was unable to cause signs of hypoglycemia in doses up to 2.4 μg/g in fasting mice, when native insulin caused 100% mortality. In neutral and acidic solutions, in absence of chaotropic agents, it behaved as the monomer, and could be separated from less iodinated, active species, that appeared as dimers, by conventional gel filtration. To generate antibodies in guinea-pigs, the tetraiodinated insulin was injected in doses three times higher than native insulin, without any harm to recipient animals. The induced antiserum was compared with antiserum generated by conventional methods in radioimmunoassay (RIA) of native insulin, and parallel curves were obtained.

KEY WORDS - Insulin Antiserum. Insulin Radioimmunoassay. Iodinated Insulin. Non-hypoglycemic Insulin.

*Correspondence Author: Dr. I. F. Heneine, Biophysics Laboratory, Department of Physiology and Biophysics, Institute of Biological Sciences, Federal University of Minas Gerais, 31 270-901 Belo Horizonte, MG, Brazil FAX: +55 (31) 441-0835.

INTRODUCTION

The preparation of antisera against insulin is a burdensome procedure, demanding measures to avoid death of inoculated animals from hypoglycemia (1-3), what actually increases the costs of production. These inconveniences could be circumvented by using an insulin derivative devoid of hypoglycemic activity, with little alteration in the immunological properties of the modified molecule. In this situation, it could be expected that a polyclonal antiserum induced by such derivative would recognise efficiently the native molecule.

In our laboratory, iodination of proteins has provided a suitable means of inactivating lethal and lesion capacity of venoms and toxins: (4-10). These iodinated derivatives behaved as good immunogens, and the antisera generated by them protected the animals against challenge with the native, lethal antigens. Iodination of allergenic proteins from extracts of *Schistosoma mansoni* did annul completely its ability to elicit immediate hypersensitivity reactions, while the capacity to induce antibodies against the unmodified preparations was retained (11). Biological activity of some naturally occurring proteins is altered after partial iodination. Rat submandibular kallikrein looses its ability to hydrolyse synthetic and natural substrates, and is devoid of oxytocic activity (12). Similar effects were noticed for tonin (13). The haptenized derivatives kept immunogenic properties, and rabbit or rat polyclonal antibodies did recognise the native molecules.

Iodination of insulin with either stable or radioactive iodine has been extensively studied, and conditions for selective incorporation of iodine are well known. It is possible to substitute almost exclusively in the A chain (3, 14-16), forming a tetraiodo derivative with two diiodotyrosil residues at Tyr-A14 and Tyr-A19. These authors also reported that the metabolic activity of the iodinated insulin decreased in proportion to the number of iodine atoms incorporated.

Therefore, a tetrasubstituted iodoinsulin was prepared with stable iodine, and it proved unable to cause hypoglycemia. In this paper we describe the iodination of insulin, separation of iodinated species, testing of residual activity, generation of an antiserum with the deactivated insulin, and the comparative performance with native-induced antiserum in radioimmunoassay (RIA).

MATERIALS AND METHODS

Insulin and animals

Bovine insulin (26 units/mg) was from BIOBRÁS S. A. Guinea- pigs (350 to 450 g) and Swiss mice (18 to 22 g) were from the animal house of the Department of Physiology and Biophysics. All animals received humanitarian treatment and number was kept to a minimum.

Iodination procedure

The stock solution of iodine monochloride was prepared as described by Hales and Randle (17). Conditions were selected as to iodinate only the A-chain. Bovine insulin was dissolved as a $5 \cdot 10^{-5}$ M solution in HCl 100 mmol/L. All iodinations were performed in an ice bath at 0° C. Volumes of 1.0 ml of the insulin solution were titrated with 2 to 8 aliquots of 25 µl of a 2 mmol/L ICl in 100 mmol/L HCl containing 1 mol/L NaCl, corresponding from 2 to 8 atoms of iodine per molecule of insulin. The measurement of substituted iodine was followed by ultraviolet differential spectroscopy (18), and in some experiments by isotopic dilution with small amounts of ^{125}I (Amersham). Mock-iodinated insulin was prepared by using the same amount of a blank solution without iodine. Both native, and mock-iodinated insulins were used as controls.

Gel filtration behaviour of native and iodinated insulins

For the separation of iodinated species and determination of molecular masses, a Sephadex G-50F column of 1.2 x 98 (cm), fluxed upwards, equilibrated with 1 mol/L acetic acid (19), or with Tris-HCl 100 mmol/L, pH 7.6, in presence or absence of 6 mol/L urea (or 4 mol/L guanidine hydrochloride), was calibrated with Blue Dextran, myoglobin (16.9 kD), cytochrome C (13.4 kD) from Sigma, and glucagon (3.5 kD), from Ely Lilly. For preparative purposes the column was scaled up to 2.5 x 64 (cm), and elution was carried out with a solution of 0.1 mol/L acetic acid plus 10 mmol/L ammonium hydroxide (pH 3.5).

The testing of hypoglycemic activity of native and iodinated derivatives

The testing for hypoglycemic activity by the mouse convulsion test was applied as follows: a) To determine the relationship between insulin dosage and clinical signs of hypoglycemia, fasting mice for 24 h in groups of 10 were individually weighed, and each mouse was injected i.p. using a Hamilton syringe, with 0.3; 0.6; 1.2 and 2.4 μg/g of native or mock-iodinated insulin for each group. b) To verify the correlation between the amount of iodine incorporation in insulin and changes in biological activity, similar groups were injected with 2.4 μg/g with insulin derivated with increasing amounts of iodine, as stated in iodination procedure.

Immunisation of guinea-pigs

The method of Yagy et. al. (2, 20) was used. In the first group, three guinea-pigs received s.c. four doses of 0.35 mg/0.5 ml of native insulin. The initial dose (day 0), was emulsified with complete Freund's adjuvant, the second dose (day 14), with incomplete Freund's adjuvant, the third (day 28) and fourth doses

(day 42) dissolved in buffer. A 10% glucose solution was furnished for 24 hours. Food was permitted freely. In the second group, with 1.05 mg/0.5 ml of native insulin, there was a high mortality rate after inoculation, impeding the obtaining of an immune serum. In the third and fourth groups, with iodinated insulin, a similar timetable and dosage was followed, but the glucose solution was omitted. Seven days after the fourth inoculation (day 49) blood was collected by cardiac puncture.

Radioimmunoassay of insulin antibodies

The radioimmunoassay for insulin antibodies was performed at the Laboratory of Quality Control of BIOBRÁS S.A., using native insulin as antigen (21). Briefly, 100 µl of a standard insulin from 2.5 to 50 µUnits/ml were mixed with 100 µl of [^{125}I]-insulin, giving about $2 \cdot 10^5$ cpm, and 100 µl of each immune serum in appropriate dilution added. After 30 min incubation at 37° C, 200 µl of dextran coated charcoal was added, tubes were swirled, and after 30 min rest at room temperature, were centrifuged, and supernatant was counted in a gamma-counter calibrated to ^{125}I.

RESULTS AND DISCUSSION

Iodine incorporation

The results of the spectrophotometric titration were concurrent with the measurement of the incorporated ^{125}I, and indicated that with addition of 4 I^+ atoms per insulin molecule, derived species with less than 4 iodine atoms incorporated were present. With 6 to 8 I^+ atoms, the level of substitution was between 4.2 to 4.8 iodine atoms/mol insulin. Our iodination results clearly repeated published procedures (3, 14-16), indicating the incorporation of 4 atoms per insulin molecule, and accordingly, in the conditions here employed, a tetrasubstitution only in the A-chain is expected

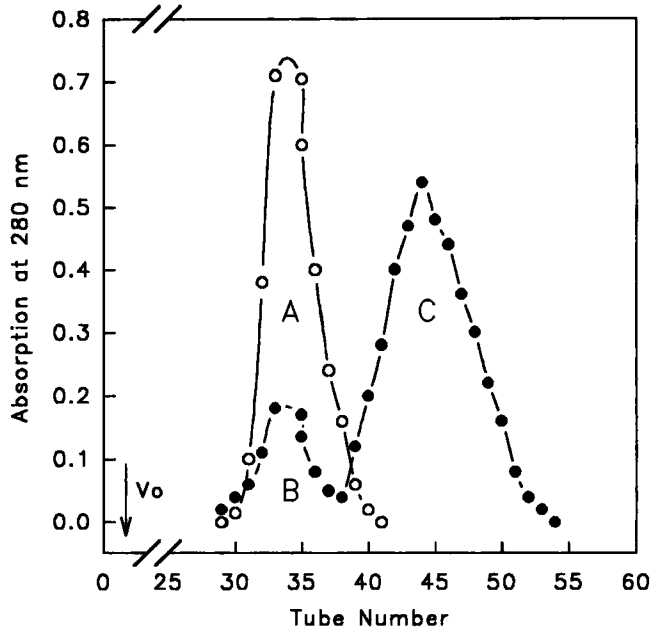

FIGURE 1 - Gel Exclusion Profile of Native and Iodinated Insulins in a Sephadex G-50F Column. (°) native insulin. (•) iodinated insulin. A- Peak of native insulin. B- Peak of insulin with less than 4 iodine atoms per mol. C- Tetraiodinated insulin.

Gel filtration

The molecular masses obtained from gel filtration corresponded to a 11.8 kD dimer for the native, mono and diiodinated insulins. The tetraiodinated molecular species appeared as a monomer with 5.7 kD, in a single symmetrical peak. The elution pattern in neutral or acidic solutions of native and iodinated insulins is shown in Fig. 1. The graphic is a composite pattern from separations in absence of chaotropic agents of native insulin, and an insulin batch titrated with 4 iodine atoms per mol. Peak A is the native insulin; peak B, is an iodinated dimer with less than four iodine atoms/mol, conserving biological activity; and peak C, the tetraiodinated monomer, inactive. The mono and diiodinated insulins almost superposes the native peak, and were not represented. It can be seen that in conventional cromatography, the tetraiodinated monomer (peak C) was well separated from the less iodinated species, except for a small zone of overlapping.

TABLE 1
RELATIONSHIP BETWEEN IODINE ADDED / INCORPORATED IN INSULIN DERIVATIVES, AND DEATHS IN CONVULSION TEST.

Experiment Number	1	2	3	4	5	6
Iodine Added (atoms I^+/molecule insulin)	0	2	4*	4*	6	8
Iodine Incorporated (spectrophotometry)	-	1.6	3.4	3.9	4.3	4.6
% Deaths	100	100	25-30	0	0	0

* Same sample. Third experiment, injected as iodinated. Fourth experiment, filtered before injection in Sephadex G-50F, to exclude the active dimer. (See Fig. 1).

Testing for hypoglycemic activity

With native or mock-iodinated insulin, the groups taking 0.3 μg/g showed only irritability, and some occasional convulsion. In the groups with 0.6 μg/g cases of irritability, spastic contractions, and deep reversible coma with a few deaths, were noticed. In the groups with 1.2 μg/g, coma was followed by deaths on 30 to 40% of mice. The groups with 2.4 μg/g showed consistently 100% of deaths. The dose of 2.4 μg/g for a 20 g mouse is equivalent to more than 2,100 I.U. for a human being of 70 kg body weight, what is beyond any tolerable limit. All obits occurred within 2 to 4 h after insulin injection. No differences in activity were noticed between native or blank-iodinated insulin, indicating that iodination was responsible for the effects observed in substituted samples.

With the modified insulins, the relationship between the amount of iodine incorporated and modifications in the biological activity is seen in Table 1. Before death, the mice showed irritability, convulsions, spastic contractions, and deep coma. No signs appeared in mice taking the tetraiodinated insulin. It is important to notice that insulin derivatives with less than four iodine atoms incorporated, proved active, as shown by results in second and third columns of Table 1. This is confirmed from experiments were the system iodinated with 4 I^+ per mol of insulin was not filtered in Sephadex G-50F (third column), or filtered (fourth column), before being injected. Published results reported that tetraiodinated insulin has still some activity (3, 16, 18). These authors did not state if, after iodination, the less

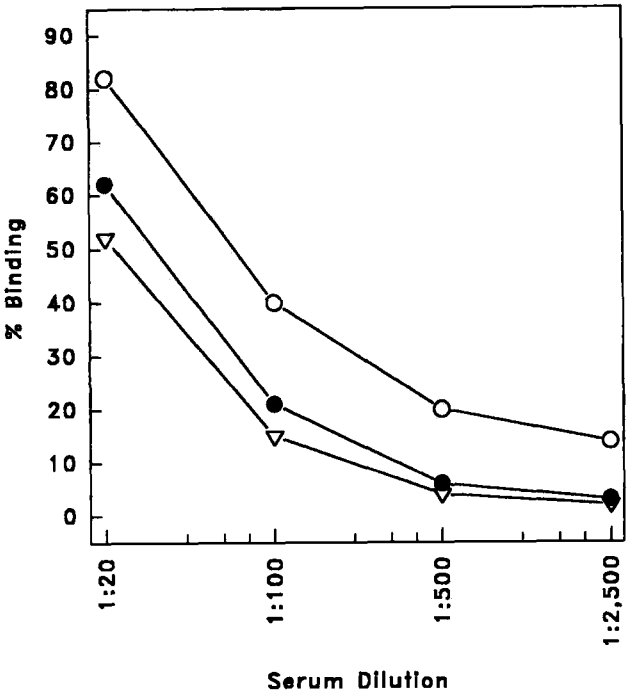

FIGURE 2- Radioimmunoassay With Guinea-pig Antisera Generated by Native and Iodinated Insulin. (○) Antiserum against iodinated insulin (group 4). (●) Antiserum against native insulin (group 1). (▽) Antiserum against iodinated insulin (group 3).

iodinated species were separated from the tetrasubstituted derivative, what could explain the differences between their findings and results here reported. An stimulation of hypoglycemic activity, with deaths appearing at earlier times than with unmodified insulin was found in samples with about 2 I^+ per mol, but with low statistical significance ($p > 0.2$).

Radioimmunoassay

In the RIA for insulin antibodies, the curves for antisera generated by native, or iodinated insulins, displayed parallel slopes (Fig. 2). These results indicated that both antisera were similar in their immunological properties. The

antibodies generated by the tetraiodinated antigen recognised the native insulin, meaning that the conformational transition induced by iodine did not cause extensive disfigurement in its epitope topology. Observations with other lightly iodinated proteins using optical rotatory dispersion (22, 23) and X-rays diffraction (24) indicated that large shape modifications are absent. A contributing fact is that the A-chain, in here modified, is not responsible for the induction of antibodies against the whole insulin molecule (20). The lower level of antibodies against native insulin ($p<0.1$) should be expected, and can be attributed to epitopes modified by the hapten. This was compensated by using the inactive insulin in a dosage 3 times higher, generating about the double amount of antibodies ($p<0.05$). Similar findings were already observed for other iodinated proteins and toxins (5,6,8,11). As the tetraiodoinsulin do not induce hypoglycemia, larger doses can be safely used to generate an antiserum. An overall conclusion is that antibodies generated by the iodinated insulin, with inherent advantages, can substitute the similar ones generated by the conventional procedures, as far as the RIA is concerned.

ACKNOWLEDGEMENTS

This paper is dedicated to the fond memory of the late Joe R. Kimmel, M.D., Ph.D., Department of Biochemistry and Molecular Biology, University of Kansas Medical Center. Support from FAPEMIG, CNPq, PRPq-UFMG, and technical assistance of Mr. Ronaldo L. Nunes, from the Biophysics Laboratory is acknowledged.

REFERENCES

1. Robinson, B.H B. and Wright, P.H. J. Guinea-pig anti-insulin serum. J. Gen. Physiol. 1961; 155: 302-10

2. Yagy, Y., Maier, P. and Pressman, D. J. Two different anti-insulin antibodies in guinea pig antisera. J. Immunol. 1962; 89: 442-51

3. Izzo, J.L., Roncone, A., Izzo, M.J. and Bale, W.F. Relationship between degree of iodination of insulin and its biological, electrophoretic, and immunochemical properties J. Biol. Chem. 1964; 239: 3747-54

4. Heneine, L.G.D., Cardoso, V., Daniel, J.P. and Heneine, I.F. Detoxification of the T_2 fraction from a scorpion (*Tityus serrulatus*, Lutz and Mello) venom by iodination and some properties of the derivatives. Toxicon,1986; 24: 501-5

5. Daniel, J.P., Heneine, L.G.D., Tavares, C.A.P., Nascimento, M.C.S. and Heneine, I.F. Generation of protective immunesera by *Crotalus durissus terrificus* venom detoxified by controlled iodination. Brazilian J. Med. Biol. Res. 1987; 20: 713-20

6. Heneine, I.F., Heneine, L.G.D., Daniel, J.P., Nascimento, M.C.S. and Rocha, O.A. Properties of protein toxins and venoms modified by controlled iodination. ACIESP, 1988; 57-II: 55-66

7 Bicalho, R.X., Rocha, O.A., Heneine, L.G.D., Magalhães, A. and Heneine, I.F. The effect of stepwise iodination on biological properties of *Bothrops jararaca* venom. Toxicon, 1990; 28: 171-9

8. Rocha, O.A., Bicalho, A.F.X., Silveira, J.N. Lopes, E.S. and Heneine, I.F. A nontoxic derivative of *Bothrops jararaca* venom suitable to generate antibodies against the native venom. Deutsche tierärtzliche Wochenschrift, 1992; 99: 143-5.

9. Soares, H.R., Higashi, H.G. and Heneine, I.F. Anatoxina tetânica por iodação controlada. XVI Congresso Brasileiro de Microbiologia. 1991; São Paulo, Abstract MMH-08.

10. Heneine, I.F., Lahmann, W.M. and Rocha, O.A. A toxoid prepared from cholera toxin by iodination. Brazilian J. Med Biol. Res. 1992; (in press)

11. Passos, R.M., Pereira, L.H., Chavez, C.O., Chamone, M. and Heneine, I.F. Iodinated soluble worm adult proteins (SWAP) preparations from *Schistosoma mansoni* are unable to induce immediate hypersensitivity reactions but retain other immunogenic properties. Brazilian J. Med. Biol. Res. 1991; 24: 787-90.

12. Feitosa, M.H., Pesquero, J.L., Oliveira, G.M.R., Beraldo, W.T. and Heneine, I.F. The action of iodinated kallikrein on the rat uterus. In: Rothschild, A.M., ed. Contributions to Autacoid Pharmacology. Basel, Birkhäuser Verlag 1992: 36: 265-70.

13 Lopes, E S., Lahman, W.M., Pesquero, J.L., and Heneine, I.F. Anticorpos contra a tonina haptenizada induzida em ratos alogênicos. (1992) VII Reunião Anual da FESBE, Caxambú, Abstract 21.23, pp. 328.,

14. Springell, P.H. An unreactive tyrosine residue in insulin and the exclusive iodination of the A chain. Nature.1961; 191: 1372-3.

15. Springell, P.H. Reaction of iodine with insulin and fibrous insulin. Biochim. Biophys. Acta. 1962; 63: 136-49.

16. Massaglia, A., Rosa, U., Rialdi, G. and Rossi, C.A. Iodination of insulin in aqueous and organic solvents. Biochem. J. 1969; 115: 11-8.

17. Hales, C.N. and Randle, P.J. Immunoassay of insulin with insulin-antibody precipitate. Biochem. J. 1963; 88: 137-46.

18. Freychet, P., Roth, J. and Neville, D.M. Jr. Monoiodoinsulin: demonstration of its biological activity and binding to fat cells and liver membranes. Biochem. Biophys. Res. Comm. 1971; 43: 400-8.

19. Kimmel, J.R. and Pollock, H.G. J. Studies of human insulin from nondiabetic and diabetic pancreas. J. Am. Diabetes Association. 1967; 16: 687-94.

20. Yagy, Y., Maier, P. and Pressman, D. Antibodies against the component polypeptide chains of bovine insulin. Science. 1965; 47: 617-9.

21. Biobrás Sociedade Anônima. Radioimunoensaio de Insulina. 1990; Procedimento Nº CA-0215-C-432. Biobrás S.A., 39 400-001, Montes Claros, MG. Brazil.

22. Perlman, R.L. and Edelhoch, H. J. The formation of diiodotyrosine in iodinated human serum albumin. J. Biol. Chem.1967; 242: 2416-22.

23. Wassarman, P.M. and Kaplan, N.O. Iodination of mucle fructose diphosphate aldolase. J. Biol. Chem. 1968; 243: 720-9.

24. Low, B.W., Potter, R. and Jackson, R.B. J. X-ray crystallographic study of the erabutoxins and of a diiodo derivative. J. Biol. Chem. 1971; 246: 4366-8.