Rev. Inst. Med. trop. São Paulo
39 (2): 119-122, março/abril, 1997

EFFECT OF β-PROPIOLACTONE TREATMENT ON THE COMPLEMENT ACTIVATION MEDIATED BY EQUINE ANTISERA.

Rosalvo GUIDOLIN (1), Josefina Farina MORAIS (1), Marco Antonio STEPHANO (1), José Roberto MARCELINO (1), Ivone Kazuko YAMAGUCHI (1) & Hisako Gondo HIGASHI (1)

SUMMARY

Reduction of complement activation through an alteration of the Fc fragment of immunoglobulins by β-propiolactone treatment was carried out in equine antisera raised against rabies virus, *Bothrops* venoms and diphtherial toxin. Results were evaluated by means of an anaphylactic test performed on guinea-pigs, and compared to the ones obtained with the same sera purified by saline precipitation (ammonium sulfate), followed or not by enzymatic digestion with pepsin. Protein purity levels for antibothropic serum were 184.5 mg/g and 488.5 mg/g in β-propiolactone treated and pepsin-digested sera, respectively. The recovery of specific activity was 100% and 62.5% when using antibothropic serum treated by β-propiolactone and pepsin digestion, respectively. The antidiphtherial and anti-rabies sera treated with β-propiolactone and pepsin presented protein purity levels of 5,698 and 7,179 Lf/g, 16,233 and 6,784 IU/g, respectively. The recovery of specific activity for these antisera were 88.8%, 77.7%, 100% and 36,5%, respectively. β-propiolactone treatment induced a reduction in complement activation, tested "*in vivo*", without significant loss of biological activity. This treatment can be used in the preparation of heterologous immunoglobulins for human use.

KEYWORDS: Heterologous antisera; Complement activation; β propiolactone; Antisera.

INTRODUCTION

The methods used for heterologous antisera purification aim to reduce the content of non-specific proteins. Heterologous antisera of higher purity have been obtained by performing enzymatic digestion of plasma proteins. Various enzymes such as trypsin, papain and pepsin have been experimentally used for this purpose [10,11].

Pepsin [12] and plasmin [13] are the most favoured enzymes in the industrial production of homologous sera. Considering both the results obtained and the required working conditions, pepsin has been the most convenient one for producing heterologous sera. [4, 10, 11]. Owing to the pepsin action of breaking the Fc region of the antibody molecule into fragments and of reducing the possibility of spontaneous formation of IgG aggregates, the number of adverse reactions when heterologous sera are administered is less than with preparations purified by non-enzymatic methods. This may be due to the presence of foreign proteins or by-products of the classical pathway of complemente cascade. Complement can also be activated by the alternative pathway[9] even when heterologous sera are submitted to different treatments such as heating, enzymatic action and others.

In an attempt to reduce complement activation by whole IgG that had not been enzymatically cleaved, STEPHAN [14] used β-propiolactone aiming to alter the structure of the human IgG Fc region. β-propiolactone reacts specifically with lysine, cystine and histidine residues. This reaction is due to the lactone ring present in β-propiolactone which can open either to the alkyl or the acyl carbon [14].

The authors have, hitherto, no knowledge of publications demonstrating the β-propiolactone anticomplementary action in equine sera. Thus, the main goal of the present work, is to study the effect of β-propiolactone on antirabies, antibothropic, and antidiphtherial sera. Sera teated with β-propiolactone were compared to the same sera purified by ammonium sulfate, followed or not by treatment with pepsin, in regard to their ability to induce anaphylaxis in guinea-pigs and to activate complement.

MATERIALS AND METHODS

Hyperimmune plasma

Plasma samples were obtained from blood collected from immunized horses at Instituto Butantan for producing antirabies, antibothropic and antidiphtherial sera.

(1) Instituto Butantan, Divisão de Desenvolvimento Tecnológico e Produção, Av. Vital Brazil 1500, 05503-900 São Paulo, Brasil.

METHODS OF PURIFICATION

Ammonium sulfate: A volume of 24% (w/v) p.a. ammonium sulfate solution (ASS) was slowly added to a volume of plasma under constant agitation. After addition of 0.3% (v/v) of toluol, the pH of the mixture was adjusted to 6.92 (with HCl 2.5 M).The mixture was then submitted to stirring during one hour at room temperature and maintained at rest overnight at 5 ± 1° C.

After eliminating the precipitate, 50% (w/v) ASS was added to the mixture until it reached a final concentration of 33%. The pH was adjusted to 6.95 (with NaOH 5N) and the mixture submitted to stirring during two hours at room temperature and maintained at rest overnight at 5 ± 1° C. The precipitate was then collected, washed twice with 30% (w/v) ASS, and dissolved in a 0.85% NaCl solution (SS) corresponding to, approximately, 50% of the plasma initial volume. Then, the material was dialysed through a Molecular Ultrafiltration System (U.F.) equipped with a 10kDa limited-cut filter until negative to the Nessler's reagent for the presence of ammonium sulfate. After the pH was adjusted to 5.4 with a 10% (v/v) solution of p.a. acetic acid, the serum was maintained at rest overnight at 5 ± 1° C and concentrated by U.F. 10 kD to a volume corresponding to one tenth the plasma initial volume.

Enzymatic Digestion: 30% (w/v) ASS was added under constant stirring to an equal volume of plasma, previously heated at 37°C. The mixture was adjusted to pH 7.2 with NaOH 5N and, after being under agitation for 3 hours followed by one night at rest at 5 ± 1° C, the precipitate was discarded. The supernatant was diluted in SS to attain a protein concentration of 2.5 - 3.0% and its pH was adjusted to 7.2 Then, solid ammonium sulfate was added for a final concentration of 28% (w/v). The mixture was stirred for one hour at room temperature and maintained at rest overnight at 5 ± 1° C. The precipitate thus obtained was dissolved again in SS until it reached a protein concentration of 2.5 - 3.0%; then, 0.22 M p.a. glycine; HCl 2.5M for adjusting the pH at 3.4; 0.2% (v/v) toluol, and 0.01 g/dl pepsin 1:10.000 (Sigma) were added. Digestions were perfomed at 27-28° C for 3 hr with constant stirring. Then, the pH was adjusted to 7.2 (with NaOH 5N) and solid ammonium sulfate was added, under constant agitation, to a final concentration of 17.5%. After stirring for 2 hours at room temperature, the precipitate was discarded. The pH of the supernatant was readjusted to 7.2 (with NaOH 5N) and solid ammonium sulfate was added for a final concentration of 30%. The mixture was stirred during one hour and kept at rest for one additional hour, at room temperature; than, the supernatant was discarded. The precipitate containing immunoglobulins was dissolved in a SS volume corresponding to the initial plasma volume; this solution was then submitted to molecular ultrafiltration through a 30 kD limited-cut filter until negative to the Nessler's reagent test for the presence of ammonium sulfate; afterwards, this solution was concentrated ten times in relation to the initial plasma volume, using the same ultrafiltration equipment.

Treatment with β-propiolactone: An aliquot of the serum, previously purified by ammonium sulfate, was diluted in 1:2 in SS. A 1:40 (v/v) β-propiolactone solution in distilled water was added under agitation to a final concentration of 0.1% of in the serum. The material was stirred for 2 hours, at room temperature, and maintained at rest overnight at 5 ± 1° C, followed by stirring for 2 hours at 37° C.

Analytical Methods

Ammonium sulfate: Concentration of ammonium sulfate was determined through conductivimetry [6].

Total proteins: Total proteins were determined by the Biuret method [7], using a wavelenght of 545 nm.

Rabies antibodies: Titers were evaluated by a Rapid Fluorescent Inhibition Microtest[3].

Bothropic antibodies: Bothropic antibodies were determined by the technique described by DALMORA et al [1].

Diphtherial antibodies: Diphtherial antibodies were determined by the Ramon's [16] basic flocculation technique.

Electrophoresis in cellulose acetate: Electophoresis were run in a cellulose acetate support and electrophoretograms were obtained according to the technique described by STEPHANO et al [15].

Anaphylactic test in guinea-pigs: Sera under test and reference horse serum were adjusted by dilution in SS to the same protein concentration. Guinea-pigs weighing 450-500 g were given a preparatory dose of 0.1 ml of each serum, subcutaneously injected in their backs. After 25 days, each guinea-pig was given a 1.0 ml unleashing-dose of the sera, by a slow intracardiac injection. The animals were observed immediately after the unleashing-dose and during the following 3 hours. Results were expressed in terms of: absence of symptoms characteristic of anaphylaxis, light symptoms (immediate or delayed), strong symptoms (immediate or delayed), and death.

Estimating percentage recovery and specific activity ratio: The percentage of specific biologic activity recovery was estimated based on the neutralizing titers observed in the initial serum (whole IgG obtained from the treatment with ammonium sulfate) and the titers obtained after the β-propiolactone or pepsin treatments. The specific activity ratio of the preparations was evaluated in IU units (anti-rabies), in mg of neutralized reference bothropic venom and in Lf unit (antidiphtherial) per g of total protein specific activity was expressed as the

$$ratio = \frac{\text{specific activity of the sample}}{\text{specific activity of the original sample}}$$

In addition, the ratio $\frac{\text{alb.} + \alpha\text{glob.}}{\beta\text{ glob.} + \gamma\text{glob.}}$ was estimated

from the electrophoresis results as an estimation of the quality of purification. A ratio ≤ 0.4 was associated with a good purification.

RESULTS AND DISCUSSION

Table 1 presents the results of anaphylactic tests observed with anti-rabies, antibothropic and diphtherial sera purified by different methods for obtaining the digested and whole IgG. The results demonstrate the relative effectiveness of the pepsin treatment on the reduction of complement activation by the classical pathway. β-propiolactone, similarly to pepsin, can also reduce efficiently the complement activation by the tested sera. Nevertheless, the β-propiolactone treatment did not alter neutralization titers, while considerable reduction was observed after treatment with pepsin.

Table 2 gives, in an abridged way, the results of electrophoretic studies, protein content and neutralizing activity of antirabies, antibothropic and antidiphtherial sera containing whole IgG, treated or not with β-propiolactone, or pepsin-digested IgG. The treatment of the anti-

TABLE 1

Results of anaphylactic tests carried out in guinea-pigs inoculated with antibothropic, anti-rabies and antidiphtherial sera containing whole IgG purified by ammonium sulfate precipitation and after treatment with β-propiolactone or pepsin.

SERUM ANTI	PROCE-DURE	Nº OF GUINEA- PIGS	SYMPTOMATOLOGY EVOLUTION
RABIES	A	4	3 with I.S. died after 3 min. 1. with D.S. recovery within 15 minutes.
	B	4	2 with D.S. recovered within 15 min. 1 died and 1 asymptomatic.
	C	4	1 with D.S. recovered within 15 min. and 3 asymptomatic.
BOTHROPIC	A	4	4 with I.S. and 3 died after 15min. 1 recovered within 15 minutes.
	B	4	3 with D.S. and 2 recovered within 15 min. 1 died and 1 asymptomatic.
	C	4	4 asymptomatic.
DIPHTHERIAL	A	4	3 with I.S. died within 13 min. 1 with I.S. recovered within 15 minutes.
	B	4	2 with D.S. died within 15 min. 2 asymptomatic
	C	4	2 with I.S. recovered after 15 min. 2 asymptomatic
REFERENCE	D	10	10 with I.S. died within 3 min.
	E	8	8 asymptomatic

A: ammonium sulfate precipitation (whole IgG); B: pepsin digested IgG; C: whole IgG treated with β-propiolactone; D: crude antibothropic or anti-rabies plasma; E: saline solution.
DS - Delayed Symptoms; IS - Immediate Symptoms.

TABLE 2

Electrophoretic studies, protein content and neutralizing activity of anti-rabies, antibothropic and antidiphtherial serum containing whole IgG treated or not with β-propiolactone and pepsin digested IgG.

		ANTI-RABIES			ANTIBOTHROPIC			ANTIDIPHTHERIAL		
PROCEDURE		A	B	C	A	B	C	A	B	C
TOTAL PROTEIN CONCENTRATION (g%)		12.41	12.32	10.76	8.20	7.8	2.56	14.72	14.02	9.75
PROTEIN FRACTION (g%)	**ALB.**	0.72	0.55	0.76	0.27	0.30	0.86	0.84	1.12	0.12
	α	5.00	1.44	1.20	1.82	1.43	0.00	2.25	2.48	1.30
	β	2.53	4.60	1.60	4.26	4.27	0.85	6.92	6.74	2.52
	γ	4.16	5.73	7.20	1.85	1.80	0.85	4.71	3.68	5.81
ALB + α/β +γ RATIO		0,86	0.19	0.22	0.34	0.28	0.51	0.27	0.35	0.17
NEUTRALIZING ACTIVITY/ml		2.027UI	2.000UI	730UI	12.7 mg	13.0 mg	7.91 mg	900UI	800 UI	700 UI
SPECIFIC ACTIVITY RATIO		1.0	0.99	0.41	1.0	1.07	1.99	1.0	0.93	1.17

A: Ammonium sulfate precipitation (whole IgG); B: whole IgG treated with β-propiolactone; C: pepsin digested

rabies, antibothropic and antidiphtherial sera with β-propiolactone did not alter significantly the content of the different protein fractions.

As shown in Table 2, by comparing the data generated with Albumin + αglobulin/β + γ globulin ratio, the treatment with β-propiolactone of antirabies, and antibothropic sera resulted in a purification degree of 0,19 and 0,28, respectively, and in a biological activity recovery of approximately 100% in both sera. The neutralizing effect of the antirabies and antibothropic sera treated by pepsin were 36.5% and 37.7% less than the samples treated by β-propiolactone. However, it should be noted that in antirabies serum 34.8% of the β-globulin was lost. As previously shown[8] the content of specific rabies antibodies in β-globulin fraction of immunized horses against rabies virus is relatively high. To minimize the risk of anaphylactic reactions the equine sera to be used in human treatments are generally digested by pepsin, but this digestion is routinely carried out in a relative short period of time (about 30 minutes) and the amount of remaining intact horse Ig may still be relatively high[5]. In order to further reduce the intact Ig level we have increased digestion time to three hours, but the electrophoretic studies show a marked loss of β + γ - globulins (27.9%) with 62.3% recovery of the original biological activity in the antibothropic serum. By comparing the data obtained with β-propiolactone or pepsin treatment of the antidiphtherial serum the differences were not significant probably due to the higher content of IgG(T) obseved in horses after several booster immunizations with toxic antigens.

RESUMO

Efeito do tratamento de antissoros equinos pela β-propiolactona na ativação do complemento.

A redução da ativação do complemento através de uma alteração do fragmento Fc das imunoglobulinas pela β-propiolactona foi obtida em soros hiperimunes equinos antivirus rábico, venenos *Bothrops* e toxina diftérica. Os resultados foram avaliados por teste de anafilaxia em cobaias, e comparados com aqueles obtidos com os mesmos soros purificados por precipitação salina (sulfato de amônio), seguidos ou não por digestão enzimática com pepsina. Os níveis de pureza protéica foram para o soro antibotrópico de 184.5 mg/g e 488.5 mg/g tratado pela β-propiolactona e digeridos pela pepsina, respectivamente. A recuperação da atividade específica foi de 100% e 62,5% no soro antibotrópico tratado pela β- propiolactona e por digestão pela pepsina, respectivamente. Os soros antidiftérico e anti-rábico tratados com β–propiolactona e pepsina apresentaram níveis de pureza protéica de 5.698 e 7.179 Lf/g, 16,233 e 6.784 UI/g, respectivamente. As recuperações da atividade específica nestes soros foram 88,8%, 77,7%, 100% e 36,5%, respectivamente. O tratamento pela β-propiolactona induziu redução de ativação do complemento, testada "*in vivo*", sem perda significante da atividade biológica. Este tratamento pode ser usado na preparação de imunoglobulinas para uso humano.

ACKNOWLEDGEMENTS

The authors thank Miss Maria Luiza Soares, for carrying out dialysis and sera concentration by U.F. and protein determinations; Mr. Silvio de Jesus, for his collaboration purifying plasma; Mrs. Maria Jose da Silva Leme and Dr. Elizabeth J. Ghiuro Valentini, for carrying out some of the necessary tests; and Mrs. Doralice Furtado Fratelli, for typing the text.

REFERENCES

1. DALMORA, S.L.; VACCARI, S.F.; SAMPEDRO, A.M. & PEREIRA, J.E. da S. - Dosagem biológica do antiveneno botrópico. **Mem. Inst. Butantan, 54**, : 21-30, 1992.

2. DICHTELMÜLLER, H. & STEPHAN, W. - Efficacy of different i.v. immunoglobulins in mouse protection test. IN: **Separation of plasma proteins. Plenary lectures and poster presented at the joint meeting of the 19th Congress of the International Society of Blood Transfusion**, Budapest, Aug. 1982. p. 149-152.

3. FAVORETTO, S.R.; CARRIERI, M.L.; TINO, M.S.; ZANETTI, C.R. & PEREIRA, O.A.C. - Simplified fluorescent inhibition microtest for the titration of rabies neutralizing antibodies. **Rev. Inst. Med. trop. S. Paulo, 35**: 171-175, 1993.

4. HARMS, A.J. - Purification of antitoxic plasma by enzyme treatment and heat denaturation. **Biochem. J., 142**: 390-397, 1948.

5. HONG, H.A.; ROOIJAKKERS, E.J.M.; KE, N.T.; GROEN, J. & OSTERHAUS, A.D.M.E. - Methods for the purification of equine rabies immunoglobulin: effects on yield and biological activity. **Biologicals, 22**: 1-6, 1994.

6. HUSON, L. & HAY, F.C. - **Practical immunology.** 3. ed. Oxford, Blackwell Scientific Publications, 1994. p.1-3.

7. ITZHAKI, R.F. & GILL, D.M. - A micro-biuret for estimating protein. **Analyt. Biochem., 9**: 401-410, 1964.

8. MIRCHAMSY, H. - Sur la preparation et la concentration du serum antirabique en Iran. **Arch. Inst. Razi, 15**, 83-116, 1963.

9. MORAIS, J.F.; DE FREITAS, M.C.W.; YAMAGUCHI, I.K. & DIAS da SILVA, W. - Snake antivenoms from by hyperimmunized horses: comparison of the properties of their whole IgG and F (ab')$_2$ fragments. **Toxicon, 32**: 725-734, 1994.

10. POPE, C.G. & STEVENS, M.F. - The action of proteolytic enzymes on the antitoxins and proteins in immune sera. III. Further studies on enzyme systems which split the antitoxin molecule. **Brit. J. exp. Path., 32**: 314-324, 1951.

11. POPE, C.G. - The action of proteolytic enzymes on the antitoxins and proteins in immune sera. II. Heat denaturation after partial enzyme action. **Brit. J. exp. Path., 20**: 201-212, 1939.

12. SCHULTZE, H.E. & SCHWICK, G. - Übe neue möglichkeiten intravenöser gammaglobulin applikation. **Dtsch. med. Wschr., 87**: 1643-1650, 1960.

13. SGOURIS, J.T. - The preparation of plasmin-treated immune globulin for intravenous use. **Vox Sang. (Basel), 13**: 71-84, 1967.

14. STHEPHAN, W. - Undegraded human immunoglobulin for intravenoms use. **Vox Sang. (Basel)**, 28:422-437, 1975.

15. STHEPHANO, M.A.; GUIDOLIN, R.; DIAS da SILVA, W. & HIGASHI, H.G. - Padronização eletroforética de soros hiperimunes de cavalos, **Mem. Inst. Butantan, 53, Bol. Biotec., 2**: 3-13, 1991.

16. WORLD HEALTH ORGANIZATION - **Manual for the production and control of vaccines.** Geneva, W.H.O., 1997. p.60-65. (BLG/UNDP/77.1).

Recebido para publicação em 15/07/1996
Aceito para publicação em 31/03/1997