Br. J. Pharmac. (1979), **67**, 173–177

THE PROTECTIVE EFFECT OF SOME PHENOTHIAZINE DERIVATIVES AGAINST STREPTOLYSIN O

D. BESLUAU, S. GAUTHIER-RAHMAN & B. HALPERN

Institut D'Immuno-Biologie (U.20 I.N.S.E.R.M. & L.A.143 C.N.R.S.), Hôpital Broussais, 96, rue Didot, 75674 Paris Cedex 14, France

1 Chlorpromazine (Cpz) and promethazine (Pmz) protected mice against lethal doses of streptolysin O (SLO) given intravenously. Three other phenothiazine derivatives had similar but lesser effects.

2 The protective effect developed slowly; maximal protection was obtained 4 h after subcutaneous injection of Cpz and Pmz.

3 The haemolytic activity of SLO *in vitro* was also inhibited by Cpz.

Introduction

Streptolysin O (SLO) is one of the exotoxins produced by haemolytic streptococci, generally belonging to groups A and C. The letter 'O' indicates oxygen-labile. The reduced, active form of SLO is haemolytic and toxic (Todd, 1939; Barnard & Todd, 1940), and as little as 0.2 μg of the purified activated toxin injected intravenously kills a laboratory mouse in less than 2 min (Alouf & Raynaud, 1973). This cytolytic protein is related to other oxygen-labile haemolysins of bacterial origin such as pneumolysin, tetanolysin and the θ toxin of *Clostridium perfringens*. The lethal effect of intravenous SLO is primarily due to its cardiotoxicity (Bernheimer & Cantoni, 1945; Kellner, Bernheimer, Carlson & Freeman, 1956; Halbert, Bircher & Dahle, 1961; Halpern & Rahman, 1968; Gauthier-Rahman, 1970). Its sites of action are cellular (Fauve, Alouf, Delaunay & Raynaud, 1966; Alouf & Raynaud, 1968a) and subcellular (Weissmann, Keiser & Bernheimer, 1963; Bernheimer & Schwartz, 1964) membranes.

Ultramicroscopy of the myocardium of mice dying 30 s after a large dose of SLO showed only changes in membrane permeability as evidenced by marked subendothelial oedema. Necrosis of myofibrils and disruption of mitochondria and other organelles develop later (Halpern, Hollman, Rahman & Verley, 1969). Phenothiazines stabilize membranes, reduce permeability (Halpern, Liacopoulos, Liacopoulos-Briot, 1959; Spirtes & Guth, 1963; Seeman, 1966; 1972), and assist the conservation of blood (Halpern, Dreyfus & Bourdon, 1950). Chlorpromazine protects human red cells against lysis by lysolecithin (Greig & Gibbons, 1956) and against hypotonic lysis (Freeman & Spirtes, 1963). The present work examines whether phenothiazines inhibit haemolysis by SLO and protect animals against the lethal effect of the toxin.

Drugs

The phenothiazine derivatives given by Rhône–Poulenc Co. Paris included chlorpromazine (4560 R.P.; 3-chloro, 10 (3′-dimethylamino propyl)-phenothiazine hydrochloride); promethazine (3277 R.P.; 10 (2′-dimethylamino propyl)-phenothiazine hydrochloride); fenethiazine (3015 R.P.; 10 (2′-dimethylamino ethyl)-phenothiazine hydrochloride); diethazine (2987 R.P.; 10 (2′-diethylamine ethyl)-phenothiazine hydrochloride); ethopropazine hydrochloride (3356 R.P.; 10 (2′-diethylamino propyl) phenothiazine hydrochloride); 3300 R.P.; 10 (3′-dimethylamino 2′-dimethyl 1, 2 propyl)-phenothiazine hydrochloride.

Mepyramine, methysergide (UML-91), atropine sulphate, ouabain and urethane were obtained from commercial sources.

Methods

Most experiments were performed on Swiss albino mice and a few on New Zealand black mice.

SLO, kindly prepared by Merieux Laboratories, Lyon, France, was partially purified freeze-dried toxin almost wholly in the oxidized state. The three batches were very similar in protein content, but differed in their LD_{50}. The bulk of the experiments was done with Batch No. 05802 and some comparative experiments with Batches 01802 and 02803.

SLO reactivated with 0.09 M mercaptoethanol (final concentration) at 37°C for 10 min had titres of 5,000 to 10,000 haemolytic units (hu) per ml. A hu is the amount of SLO required to lyse 50% of the red cells in 0.5 ml of a standardized suspension (Rahman, Rebeyrotte, Halpern & Besluau, 1969; 1971). As haemolytic titres of SLO often changed, mostly increased,

0007-1188/79/100173-05$01.00

during the first few hours after activation, it was activated 3 to 4 h before injection, immediately titrated and then kept in an ice-bath. It was titrated again just before intravenous injection in mice, and in some cases, also at the end of the injections.

A dose of 50 hu/g body weight was used as the standard challenge. This represents a little less than 3 LD_{50}, the LD_{50} of batch 05802 being 17.5 hu/g. SLO was injected in the dorsal vein of the penis in males and in one of the tail veins in females.

Electrocardiographic study

Needle electrodes were firmly introduced into the fore paws and hind legs of the mouse which was then allowed to enter a small cardboard box. If handled gently in a quiet room, the mouse once inside the dark box moved only little and it was possible, with caution, to take brief recordings every few minutes on a Sanborn 150 preamplifier over considerable periods of time. Interference was removed by connecting the metallic operating table to the Sanborn apparatus with suitable leads. Leads 1, 2 and 3 corresponding to those used in man, were studied in normal animals and lead 2 (right fore paw, left hind leg) used thereafter, the voltages obtained being highest in this lead. Calibration was 1 mV: 10 mm and chart speed 100 mm per s. ECG recordings were read with the help of caloptic magnifying spectacles.

Mice were studied without anaesthesia, under urethane anaesthesia, after intravenous injection of atropine sulphate (10 μg/g), after various doses of phenothiazine drugs or after phenothiazine drugs followed by intravenous SLO. The sedative effect of the latter drugs made electrocardiography relatively easy during day 1 of the experiments. On day 2 or later, some mice had to be given light ether anaesthesia.

Haemolysis experiments

A standardized suspension of fresh thrice-washed rabbit red cells was adjusted so that 0.5 ml of the suspension when lysed with 4.5 ml of distilled water had an optical density of 0.50 at 540 nm. This represents 100% lysis in a final volume of 5 ml. As in haemolysis experiments 0.5 ml of the suspension was diluted to 2.5 ml, an optical density of 50 meant 50% haemolysis. Such standardized suspensions were found to contain 2.25 ($\pm$0.37) $\times$ 10^8 red cells per ml. Three types of experiments were performed: (1) The haemolytic activities of the phenothiazines studied were determined. Serial dilutions of drug in 2 ml volumes of buffer were mixed with 0.5 ml of the red cell suspension and incubated at 37°C for 25 min. After centrifugation the optical density of the supernatants was read at 540 nm and the concentration causing 50% haemolysis (optical density 0.50) was determined graphically for each drug. (2) The inhibitory effect of chlorpromazine was shown by making serial dilutions of SLO in 2 ml of buffer solution containing different concentrations of drug, one series for each drug concentration. A volume (0.5 ml) of the standardized red cell suspension was added to each tube. After 30 min at 37°C and centrifugation, haemolysis was measured as described. The SLO titre was defined as the reciprocal of the dilution of SLO alone (control) giving an optical density of 0.50. Inhibition of lysis was expressed as lower SLO titres. Percentage inhibition was calculated from the difference between the control titres of SLO alone and of SLO in presence of drug. (3) The effect of drugs on the fixation of SLO on red cells was studied. A standardized suspension of rabbit red cells was exposed to SLO at a final concentration of 25 hu/ml at 4°C with or without drug (promethazine, final concentration 0.5 μg/ml). After 1.5, 5 and 10 min, 2.5 ml of each mixture was centrifuged and the SLO titre in the supernatants determined. A SLO solution of 25 hu/ml kept at 4°C was titrated simultaneously.

Results

Protective effects of promethazine (Pmz) and chlorpromazine (Cpz)

Groups of 10 to 15 mice pretreated with different doses of Pmz and Cpz and challenged with a standard dose of SLO 50 hu/g (about 3 LD_{50}) were protected against the lethal effect of the toxin. Doses of Pmz between 15.6 μg/g to 250 μg/g were tried. The largest dose was found to be toxic. Good protection was obtained with as little as 31 μg/g (20 mice) but not with 15 μg/g (15 mice). Cpz conferred an even greater and more lasting protection than Pmz in the same doses. All of 20 mice pretreated with Cpz 62.5 μg/g subcutaneously survived challenge with SLO 50 hu/g at 4 h and 24 h; 7 of 8 mice (87.4%) were still protected at 48 h and 2 of 8 (25%) at 72 h. By 96 h the protective effect of Cpz had completely disappeared (5 mice).

After treatment with Pmz, 50 hu/g was the maximal dose of SLO against which the mice were protected, whereas after Cpz 1 of 5 mice survived a challenge with 100 hu/g when given at 4 h, 24 h or 48 h after the drug, and in 2 death was delayed. No mouse survived challenge with 200 hu/g (12 LD_{50}).

A group of 5 mice pretreated with Cpz 62,5 μg/g and surviving a first challenge with 50 hu/g of SLO on day 1 could tolerate a second challenge with the same dose at 24 h; 3 of 5 mice survived a third challenge at 48 h, but succumbed after a fourth at 120 h. By 48 h, these mice had received a total of about 9 LD_{50} of SLO.

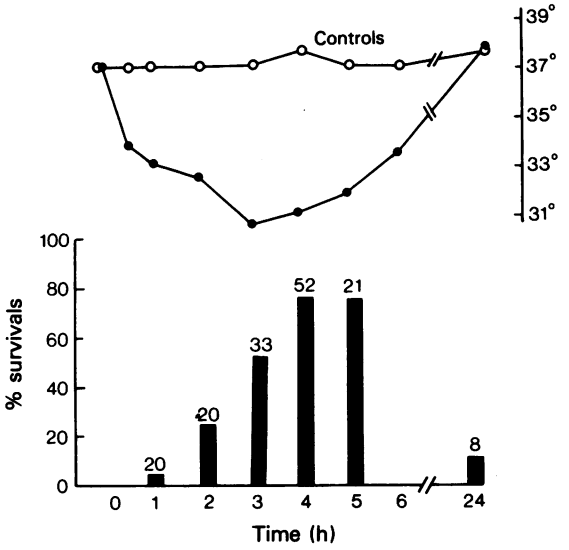

Figure 1 The effect of the interval (abcissa scale) between promethazine (Pmz) treatment and streptolysin O (SLO) challenge on the survival rate of Swiss mice (left ordinate scale). Mice were challenged after Pmz (62.5 μg/g s.c.) with intravenous SLO 50 hu/g. The figures on the top of the columns indicate the number of mice tested. The upper graph shows the mean rectal temperatures (right ordinate scale) of five mice at 0 to 24 h after Pmz treatment (●) and of five control mice (○). Maximal protection did not coincide with maximal hypothermia.

Figure 1 shows the progressive increase and duration of resistance to SLO after protective doses of Pmz. The figures are for mice that survived indefinitely. Within the dose range tested the time of challenge appeared to be paramount. Even the larger doses of the drugs provided little or no protection in the first 90 min after injection. By 2 h definite protection was present and was maximal by 4 h with a survival rate of 77%, which declined at 24 h to 12%. It was found that even among mice that died, survival time was prolonged in many cases from a few minutes to less than 24 h. Mice that died showed all the symptoms of SLO toxicity with characteristic electrocardiographic changes of arrhythmia, heart block and low voltage potentials. Pulmonary oedema and thrombosis of the great vessels and chambers of the heart was observed in many cases.

Electrocardiographic study of protected mice

Treatment with Pmz alone led to a dose-dependent increase of the PR interval, which was prolonged from normal 37.1 ± 4.6 milliseconds (ms) (mean ± s.e. mean of 25 mice) to 42.0 ± 6.4 ms and to 45.8 ± 10.1ms, respectively in 10 mice treated with 31 μg/g and 16 mice treated with 62.5 μg/g 3.5 h earlier. This increase was significant, with $P < 0.001$ for the larger dose, and $P < 0.02$ for the smaller one. It was no longer present 24 h after the drug, and was not observed after treatment with Cpz (10 mice).

The ECG of treated mice following intravenous injection of SLO remained apparently normal in survivors; only in two mice that survived indefinitely was temporary arrhythmia observed. However, a temporary mean loss of voltage of the R wave developed shortly after intravenous injection of SLO in mice pretreated with Pmz. Thus 30 min after SLO, 5 mice pretreated with Pmz 31 μg/g had a mean R wave of only 0.425 ± 0.221 (mean ± s.e. mean) millivolts (mV) as compared to 0.800 ± 0.221 mV before the injection ($P < 0.05$). Loss of voltage was still present at 90 min in 6 mice pretreated with Pmz 62.5 μg/g, the mean R being 0.575 ± 0.199 mV as compared to 0.833 ± 0.183 mV before the injection of SLO ($P < 0.05$). By 24 h voltage had returned to normal.

Other phenothiazine derivatives

The protective effect of the other phenothiazine derivatives studied was less than that of Cpz and Pmz when given to groups of 10 mice at a dose of 62.5 μg/g subcutaneously 4 h before SLO. In these conditions, 10/10 mice survived after R.P. 4560, 7/10 after R.P. 3277, 5/10 after R.P. 3015, 2/10 after R.P. 2987 and 0/10 after R.P. 3300. However, 125 μg/g of R.P. 3300 given 24 h before SLO completely protected mice. The protective effect of the derivatives corresponded fairly closely to their haemolytic effect which was most pronounced with R.P. 4560, 3300 and 3277.

Other drugs

No protection was obtained with mepyramine (40 μg/g i.p.) apart from prolonging survival from 1.25 ± 0.05 min to 3.49 ± 2.50 min (15 mice). Definite protection was seen with UML-91 (21 μg/g i.p.) with 16.8% of survivals in 59 mice. UML-91 also halved the incidence of thrombosis in the heart chambers and great vessels of mice dying after intravenous SLO. 5-Hydroxytryptamine 10 μg/g subcutaneously twice a day did not cause a significant increase in SLO toxicity, shortening mean survival time to 1.04 ± 0.11 min. (7 mice).

Sedation by urethane, 1.25 mg/g i.p. prolonged mean survival time to 5.50 ± 1.96 min (10 mice). Atropine sulphate, 10 μg/g (9 mice) and ouabain 5 μg/g (10 mice) had no effect.

In vitro *experiments*

Pmz (0.5 μg/ml) did not prevent the fixation of SLO to red cells in standard suspensions at 4°C. The con-

centration of SLO (25 hu/ml) in the supernatants of a mixture of red cells and SLO fell to 1.4 hu/ml in 90 s. In the presence of Pmz, SLO fell to 1.7 hu/ml. That the SLO had not been destroyed, but taken up by the red cells was shown by the lysis of the cell pellets when resuspended in buffer at 37°C, as well as by the practically unchanged titre, 24 hu/ml, of a control solution of SLO 10 min after the beginning of the experiment.

Inhibition of haemolysis

Non-haemolytic concentrations of Cpz (5 μg to 20 μg/ml) increased lysis of red cells by SLO. Concentrations of 0.1 μg to 2.5 μg/ml had small and variable effects. After 20 min of prior contact of red cells at 4°C with 2.5 μg/ml Cpz, the haemolysis produced by serial dilutions of SLO was inhibited by 19% ($P < 0.05$), after 40 min by 20.2% ($P < 0.02$), after 24 h by 24.2% ($P < 0.02$), and after 72 h by 24.6% ($P < 0.02$). This inhibitory effect was less with 1 μg/ml of Cpz and absent with 0.6 μg/ml.

Discussion

When given subcutaneously in doses of 31.5 μg to 62 μg/g 4 h before challenge with 3 LD_{50} of SLO intravenously, Cpz protected all, and Pmz 77% of treated mice. Of 24 other drugs tested by Halbert, Bircher & Dahle (1963), only the lysergic acid derivative UML-491 was found to have a slight though significant effect against SLO.

The protective effect develops after a latent period which cannot be attributed to slow absorption of the drugs, as other effects such as hypothermia and sedation are almost maximal by 1 to 2 h after subcutaneous injection.

The sedative, hypothermic and antihistaminic properties of these drugs are not responsible for the observed protection. The powerful antihistamine mepyramine provided no protection. Hypothermia was absent with lower protective doses of Cpz, and protection lasted longer than hypothermia and sedation. Moreover sedation by urethane had no protective effect. Sedation with barbiturates increases SLO toxicity (Halbert *et al.*, 1963) probably by depressing the cardiovascular system.

The toxic effects of intravenous SLO on the heart and the vascular and pulmonary endothelium were prevented by Cpz and Pmz, probably by their stabilizing effect on cell membranes. Pmz did not prevent the fixation of SLO on to the red cell membrane *in vitro*. This fixation is practically instantaneous even at 4°C, though lysis only occurs at higher temperatures (Alouf & Raynaud, 1968b). The changes induced by Cpz in the cell membrane which prevent the action of SLO seem to require some time to develop.

SLO is produced in haemolytic streptococcal infections and the antistreptolysin O (ASO) titre in serum almost invariably rises. Although other immunological mechanisms have been proposed for the pathogenesis of rheumatic fever (Goldstein, Halpern & Robert, 1967; Kaplan, 1968; 1976, Zabriskie & Freimer, 1968; Zabriskie, 1976) the direct toxic effect of SLO on the heart has never been excluded (Halbert, 1970). The ECG anomalies seen frequently in rheumatic carditis resemble those seen after intravenous injection of SLO in the mouse, rat and rabbit. The marked protective effect of Cpz and Pmz observed *in vivo* in the mouse might provide a rationale for the prophylactic use of these familiar drugs in the course of streptococcal infections, especially in children.

We are deeply grateful to Dr J.E. Alouf for helpful advice and discussion and for critical reading of the manuscript, and to Mr G. Leca for the statistical analysis.

References

ALOUF, J.E. & RAYNAUD M. (1968a). Action de la streptolysine O sur les membranes cellulaires. I. Fixation sur la membrane erythrocytaire. *Annls Inst. Pasteur, Paris*, **114**, 812–827.

ALOUF, J.E. & RAYNAUD, M. (1968b). Action de la streptolysine O sur les membranes cellulaires. II. Cinétique de la lyse erythrocytaire; *Annls Inst. Pasteur, Paris*, **214**, 97–121.

ALOUF, J.E. & RAYNAUD, M. (1973). Purification and properties of streptolysin O *Biochimie*, **55**, 1187–1193.

BARNARD, W.G. & TODD, E.W. (1940). Lesions in the mouse produced by streptolysin O and S. *J. Path. Bact.*, **51**, 43–47.

BERNHEIMER, A.W. & CANTONI, G.L. (1945). The cardiotoxic action of preparations containing the oxygen–labile hemolysin of streptococcus pyogenes. I. Increased sensitivity of the isolated frog's heart to repeated application of the toxin. *J. exp. Med.*, **81**, 295–306.

BERNHEIMER, A.W. & SCHWART, Z.L.L. (1964). Lysosomal disruption by bacterial toxins. *J. Bact.*, **87**, 1100–1104.

FAUVE, R.M., ALOUF, J.E., DELAUNAY A. & RAYNAUD, M. (1966). Cytotoxic effects '*in vitro*' of highly purified streptolysin O on mouse macrophages cultured in a serum-free medium. *J. Bact.*, **92**, 1150–1153.

FREEMAN, A.R. & SPIRTES M.A. (1963). Effects of chlorpromazine on biological membranes. II. Chlorpromazine induced changes in human erythrocytes. *Biochem. Pharmac.*, **12**, 47–53.

GAUTHIER-RAHMAN, S. (1970). Contribution à l'étude expérimentale de quelques effets biologiques de la streptolysine O. Thesis Paris.

GOLDSTEIN, I., HALPERN, B. & ROBERT, L. (1967). Immunological relationship between streptococcus A polysaccharide and the structural glycoproteins of heart valve. *Nature*, **213**, 44–47.

GREIG, M.E. & GIBBONS, A.J. (1956) The effect of phenothiazine drugs on the removal of cholinesterase from erythrocytes and on hemolysis by lysolecithin. *Archs Biochem. Biophys.*, **61**, 335–342.

HALBERT, S.P. (1970). Streptolysin O. In *Microbial Toxins*. ed. Montie T.C., Kadis S., & Ajl S. L., Vol. 3, pp. 69–98. New York: Academic Press.

HALBERT, S.P., BIRCHER, R. & DAHLE E. (1961). The analysis of streptococcal infections. V. Cardiotoxicity of streptolysin O for rabbits '*in vivo*' *J. exp. Med.*, **113**, 759–784.

HALBERT, S.P., BIRCHER, R. & DAHLE E. (1963). Studies on the mechanism of the lethal toxic action of streptolysin O and the protection by certain anti-serotonin drugs. *J. Lab. clin. Med.*, **61**, 437–452.

HALPERN, B., DREYFUS, B. & BOURDON G. (1950). Influence favorable sur la conservation du sang des dérivés de la phénothiazine. *Presse Médicale*, **58**, 1151–1153.

HALPERN, B.N., HOLLMAN, K.H., RAHMAN, S. VERLEY, J.M. (1969). Submicroscopical lesions produced by streptolysin O '*in vivo*' *Israel J. Med. Sci.*, **5**, 1138–1148.

HALPERN, B.N., LIACOPOULOS, P. & LIACOPOULOS-BRIOT, M. (1959). Recherches sur les substances exogènes et endogènes agissant sur la perméabilité capillaire et leurs antagonistes. *Archs Int. Pharmacodyn.*, **119**, 56–101.

HALPERN, B. & RAHMAN S. (1968). Studies on the cardiotoxicity of streptolysin O. *Br. J. Pharmac. Chemother.*, **32**, 441–452.

KAPLAN, M.H. (1968). Multiple nature of the cross-reactive relationship between antigens of group A streptococcus and mammalian tissue. In *Current Research in Group A Streptococcus*. ed. Caravano, R. p. 139. Amsterdam: Excerpta Medica Foundation.

KAPLAN M.H. (1976). Autoimmunity in rheumatic fever. Relationship to streptococcal antigens cross-reactive with valve fibroblasts, myofibres and smooth muscle. In *Infection and Immunology in the Rheumatic Diseases*. ed. Dumonde, D.C. p. 113 Oxford, London, Edinburgh: Blackwell Scientific Publications.

KELLNER, A. BERNHEIMER, A.W., CARLSON, A.S. & FREEMAN, E.B. (1956). Loss of myocardial contractibility induced in isolated mammalian hearts by streptolysin O. *J. exp. Med.*, **104**, 361–373.

RAHMAN, S., REBEYROTTE, P., HALPERN, B. & BESLUAU D. (1969). Kinetics of streptolysin O reactivation by different thiols. *Biochem. biophys. Acta.*, **177**, 658–660.

RAHMAN-GAUTHIER, S., REBEYROTTE, P. & BESLUAU, D. (1971) La réactivation de la streptolysin O par différents thiols et son incidence sur le dosage des antistreptolysines. *Revue Immunol., Paris*, **35**, 243–258.

SEEMAN, P. (1966). Erythrocyte membrane stabilization by local anaesthetics and tranquillizers. *Biochem. Pharmac.*, **15**, 1753.

SEEMAN, P. (1972). The membrane actions of anaesthetics and tranquillizers *Pharmac. Rev.*, **24**, 583–655.

SPIRTES, M.A. & GUTH, P.S. (1963). Effects of chlorpromazine on biological membranes. I. Chlorpromazine induced changes in liver mitochondria *Biochem. Pharmac.*, **12**, 37–46.

TODD, E.W. (1939). Lethal toxins of haemolytic streptococcia: a serological investigation. *J. Hyg.*, **38**, 1–11.

WEISSMAN, G., KEISER, H. & BERNHEIMER A.W. (1963). Studies on lysosomes. III. The effects of streptolysins O and S on the release of acid hydrolases from a granular fraction of rabbit-liver. *J. exp. Med.*, **118**, 205–222.

ZABRISKIE J.B. & FREIMER E.H. (1968). An immunological relationship between the group A. Streptococcus and mammalian muscle. *J. exp. Med.*, **124**, 661.

ZABRISKIE J.B. (1976). Rheumatic fever: a streptococcal induced autoimmune disease? In *Infection and Immunology in the Rheumatic diseases*. ed. Dumonde, D.C. p. 97. Oxford, London, Edinburgh: Blackwell Scientific Publications.

(*Received July 19, 1978.*
Revised January 29, 1979.)