Polyene antibiotics in assessing significance of antistreptolysin O activity

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SUMMARY Antistreptolysin O activity in serum is due either to antibody or to altered lipoprotein molecules. The latter can be inhibited by performing antistreptolysin tests using a polyene antibiotic such as amphotericin B as diluent.

Serum antistreptolysin O (ASO) activity is usually due to antibody. However, a second mechanism involves inhibition of streptolysin by cholesterol. The latter has been recorded in patients with jaundice (Badin *et al.*, 1962), in nephrosis (Stollerman, 1954), and in patients with staphylococcal infections (Watson and Kerr, 1975). The latex particle test has been thought to give positive results only with antibody but we have shown that false-positive results can arise (Watson *et al.*, 1977). Activity associated with cholesterol can be removed by precipitating the pre-beta- and beta-lipoprotein fractions with 10% dextran sulphate. This activity, which we have labelled antistreptolysin factor (ASF), results from alterations of lipoprotein molecules from the action of cholesterol esterase (Watson and Kerr, 1976). Since preliminary removal of lipoproteins from serum is time consuming we have utilised the known ability of polyene antibiotics to bind to cell membrane cholesterol as a method for distinguishing ASO from ASF activity.

Material and methods

Routine serum samples showing antistreptolysin titres greater than 240 Todd units/ml by the standard haemolysis inhibition test were stored at -20°C . Commercial latex test kits were used (Hoechst AG). Agglutination was tested for after absorption of the serum with a concentration of streptolysin sufficient to remove up to 200 Todd units/ml.

ANTISTREPTOLYSIN TESTS

A microtitre modification of the standard method

of Rantz and Randall (1945) was employed. Serum dilutions in 0.05-ml volumes were placed in round bottomed well plates. Dilutions were made in buffered saline (pH 6.5) containing 8.42 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 8.95 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, and 4.25 g of NaCl per litre. Streptolysin O was added in 0.025-ml volumes containing 1.0 unit/ml and trays were incubated for 15 minutes at 37°C . Washed human red cells, 5% concentration, were added in 0.025-ml amounts and plates were reincubated for a further 45 minutes. End points were taken as 50% haemolysis.

POLYENE ANTIBIOTICS

Amphotericin B was dissolved in sterile distilled water at a concentration of 6250 units/ml. This stock solution was further diluted by adding one part to three parts of phosphate buffered saline (pH 6.7). This resulted in a fine precipitate of amphotericin, but this did not interfere with subsequent investigations.

PREPARATION OF ANTISTREPTOLYSIN FACTOR (ASF)

ASF activity was produced by growing *Staphylococcus aureus* (NCTC strain 7121, Wood 46 strain) in 10 ml of a 60/40 v/v mixture of sterile horse serum and brain heart infusion broth at 37°C for four days with continuous shaking. After removing organisms by centrifugation the lipoprotein fraction was precipitated by adding 0.02 ml of 10% dextran sulphate and 0.1 ml of M CaCl_2 to each millilitre of supernatant fluid. The precipitate was removed after standing overnight at 4.0°C , redissolved in distilled water, and dialysed against running tap water for 18 hours.

Lipoproteins were precipitated from latex-positive and latex-negative sera in the same way, a procedure which does not affect antibody titre.

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Results

DISTRIBUTION OF ANTISTREPTOLYSIN ACTIVITY

Table 1 shows the relationship between positive or negative latex tests and presence of antistreptolysin activity in either the supernatant antibody containing fraction of serum or in the lipoprotein moiety.

Table 1 *Distribution of antistreptolysin activity in sera before and after precipitation of beta-lipoproteins*

Serum	Latex test	Antistreptolysin titre of:		
		Whole serum	β -lipoprotein	Supernatant
1	+	1280	20	1280
2	+	2560	40	2560
3	+	640	20	640
4	-	2560	2560	40
5	-	1280	1280	40
6	-	1280	1280	20

All activity in latex-positive sera was in the antibody-containing supernatant fraction whereas activity in latex-negative sera was found in the lipoprotein fraction.

OPTIMUM CONCENTRATION OF AMPHOTERICIN B

The optimum amount of amphotericin B needed to inhibit ASF activity was determined in a series of chequer board type titrations of ASF prepared from horse serum. Dilutions of amphotericin and ASF were reacted at 4.0°C for 10 minutes before the addition of streptolysin solution. We have shown previously that binding of exposed cholesterol molecules in lipoprotein fragments to streptolysin is almost instantaneous. Binding of amphotericin is equally quick. Inhibition of ASF activity is shown in Table 2 for one particular experiment.

Table 2 *Chequer board titration of ASF against dilutions of amphotericin B*

	Final dilutions of amphotericin B						
	1/4	1/8	1/16	1/32	1/64	1/128	Nil
Final dilutions of ASF							
1/20	-	+	+	+	+	+	+
1/40	-	-	-	+	+	+	+
1/80	-	-	-	-	-	-	+
1/160	-	-	-	-	-	-	+
1/320	-	-	-	-	-	-	+
1/640	-	-	-	-	-	-	+
1/1280	-	-	-	-	-	-	+
nil	-	-	-	-	-	-	-

- = Haemolysis. + = No haemolysis.
Titre of ASF = 1280.

The required amount of amphotericin was taken as that needed to reduce the original ASF titre of

1280 to 80 and corresponded to a 1/128 dilution, or about 49 units.

EFFECT OF AMPHOTERICIN B ON SERA

Test sera were mixed with aliquot 1.0-ml volumes of amphotericin B (final concentration of amphotericin B 100 units/ml) and incubated at room temperature for 20 minutes. Control samples diluted in buffered saline were included. Dilutions were then made in microtitre trays, the diluent being either amphotericin B in buffer at the same concentration or buffered saline itself. Antistreptolysin activity was then detected as before. Titres due to antibody in latex-positive sera were unaffected by amphotericin B, but titres of latex-negative sera were almost completely abolished in the presence of amphotericin as diluent. For example, no serum with a titre greater than 2560 gave a titre greater than 40 in amphotericin B, and further investigation of these sera after removal of the beta-lipoproteins with dextran sulphate showed that the low residual titres were due to small amounts of antibody. When latex-negative sera preincubated with amphotericin B were diluted in buffered saline the final antistreptolysin titre was identical with that of serum preincubated with saline, suggesting that the amphotericin-lipoprotein complex is easily reversed.

OTHER POLYENE ANTIBIOTICS

Two other polyenes, nystatin and pimarin, were investigated. Both inhibited ASF in the same way as amphotericin but were less convenient to use because of problems of initial solubility.

Discussion

During an investigation of the mechanisms of non-antibody inhibition of streptolysin O activity we found that antistreptolysin titres greater than 200 Todd units/ml were common, but such sera give negative latex tests. We have found these in some patients with rheumatoid arthritis, in staphylococcal infection, in one patient with brucellosis, and in another with *Bacteroides fragilis* infection (Watson and Kerr, 1975). In all of these the activity is in the lipoprotein fraction and is removed with 10% dextran sulphate.

We have also shown that similar activity is generated in sera incubated with *Staph. aureus* or *Pseudomonas aeruginosa* strains (Watson and Kerr, 1976). Here the mechanism involves cholesterol esterase production, possibly in association with proteolytic enzyme action. This results in the formation of peptide fractions of prebeta- and beta- lipoproteins with molecular weights between 25 000 and 100 000 where the attached cholesterol

molecules are spatially orientated so as to be capable of binding to streptolysin. The low level of ASF activity in normal sera seems to be due to this mechanism and probably reflects endogenous esterase function concerned in normal catabolic turnover of lipoproteins. Raised ASF serum levels may then be due to increased endogenous esterase activity or, possibly, as in the case of staphylococcal infection, to exogenous esterase. The former situation can arise when binding of toxin—for example, streptolysin O or other oxygen-labile related haemolysins—to the cholesterol moiety of lipoprotein peptide fragments results in a feed-back mechanism to increase esterase production. We believe the evidence suggests that lipoprotein peptide cholesterol plays a role as a defence mechanism against the action of such toxins and that this is available before antibody formation gets under way.

Our procedures to differentiate ASO from ASF activity in serum lead us to believe that many reports of raised antistreptolysin titres are wrongly interpreted as being due to antibody, and therefore suggestive of streptococcal infection, when in fact they are due to ASF. It seems necessary to distinguish between them. Probably earlier reports of raised antistreptolysin activity in rheumatoid disease

have been wrongly ascribed to streptococcal infection.

References

- Badin, J., Cabau, N., Lévy, C., and Cachin, M. (1962). L'inhibition non spécifique de la streptolysine O dans les maladies du foie: sa signification et son intérêt clinique. *Annales de Biologie Clinique (Paris)*, **20**, 525-541.
- Rantz, L. A., and Randall, E. (1945). A modification of the technic for determination of the antistreptolysin titre. *Proceedings of the Society of Experimental Biology and Medicine*, **59**, 22-25.
- Stollerman, G. H. (1954). The use of hemolysin inhibition in the study of experimental and clinical hyperlipemia: the non-specific inhibition of streptolysin O by serum lipoproteins. *Journal of Clinical Investigation*, **33**, 1233-1241.
- Watson, K. C., and Kerr, E. J. C. (1975). Functional role of cholesterol in infection and autoimmunity. *Lancet*, **1**, 308-310.
- Watson, K. C., and Kerr, E. J. C. (1976). Studies on anti-streptolysin O activity generated in serum by micro-organisms. *British Journal of Experimental Pathology*, **57**, 105-113.
- Watson, K. C., Kerr, E. J. C., and Drummond, J. (1977). Antistreptolysin activity in staphylococcal infection. *Lancet*, **1**, 651-652.