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INTERACTION OF STREPTOLYSIN O WITH STEROLS

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SUMMARY

A quantitative study of the specific inhibitory power of cholesterol and other sterols on the hemolytic properties of streptolysin O is reported. This streptococcal exocellular protein is a cytolytic toxin which disrupts cytoplasmic membranes of eukaryote cells. The structural characteristics, particularly the stereochemical ones required for a steroid molecule to inhibit the cytolytic activity of streptolysin O, have been investigated in detail. By immunodiffusion techniques, in agar gel plates or tubes containing sterols, the formation of hydrophobic complexes between streptolysin O and inhibitory steroids, but not non-inhibitory steroids except lanosterol, is shown. Upon interaction with inhibitory steroids streptolysin O loses its immunoreactive properties towards neutralizing and precipitating homologous antibodies.

An interpretation of the mechanism of biomembrane disorganization by streptolysin O is discussed in the light of its steroid binding properties.

INTRODUCTION

Streptolysin O is a lethal and cytolytic streptococcal exocellular protein of about 60 000 daltons [1, 2]. This toxin lyses erythrocytes and many other animal cells by disruption of the cytoplasmic membrane and those similar surrounding intra-cellular organelles [3] following the binding of reduced active toxin on cell surface [4].

It has long been known that cholesterol and related sterols possessing a 3 β-hydroxy group and side chain, inhibit the lytic activity of streptolysin O [5–8]. Inhibition is also observed with cholesterol-containing material such as serum β-lipoproteins [7, 9], lipid extracts from skin [10] or erythrocytes [4, 11] and artificial liposomes composed of both lecithin and cholesterol [12] but not of lecithin alone.

The same sterols inhibit the cytolytic activity of the other thirteen streptolysin O-like toxins (cereolysin, pneumolysin etc.) [13] as well as that of various saponins [14] and polyene antibiotics [15].

Several lines of evidence suggest that all these agents exert their cytolytic properties by interaction with cell membrane cholesterol [13]. Previous work has

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shown that cholesterol aqueous emulsions no longer inhibit erythrocyte lysis after binding streptolysin O on cell membrane [4]. On the other hand, filipin and saponin interfere with toxin binding on erythrocytes or on cholesterol-containing liposomes [12]. Cholesterol is a permanent and ubiquitous constituent of the lipid bilayer in the membrane of eukaryote cells but lacks in bacteria and most prokaryote cells [16] which are insensitive to both streptolysin O and polyenes [13]. The latter are known to form complexes in vitro with cholesterol and related sterols in a stoichiometrically and stereochemically defined manner [15, 17]. Exogeneous sterols very likely inhibit lysis by streptolysin O or polyenes by preventing competitively the binding of these agents on membrane cholesterol.

A quantitative study of streptolysin O-sterols interaction in vitro, performed with highly purified toxin and its effects on the cytolytic properties and immunochemical reactivity of the toxin, are reported here. It may prove useful for a better understanding of the mechanism of the membrane-disrupting action of streptolysin O and as a probe for the investigation of the molecular organization of biomembranes. We demonstrate in this study the formation of insoluble complexes between streptolysin O and sterols complying with strict structural and stereochemical characteristics. We also discuss the consequences of the steroid-binding properties of the toxin at the level of the phospholipid-cholesterol bilayer in cytoplasmic membranes.

MATERIALS AND METHODS

Streptolysin O and other streptococcal proteins. A highly purified preparation (P_1) homogeneous by immunodiffusion and disc electrophoresis tests obtained as described previously [1] was used to avoid eventual interference of other streptococcal exoproteins with sterols. A less purified preparation (P_2), containing both streptolysin O and nicotinamide adenine dinucleotide (NAD)-glycohydrolase, and a crude fraction (F_1) containing these proteins and seven to ten other streptococcal exoproteins [1], were used in some experiments to demonstrate the specificity of streptolysin O reaction with sterols.

Toxin preparations were activated by reduction with cysteine [18] and titrated about $5 \cdot 10^5$ hemolytic units/ml. They were diluted to the required titers with 0.15 M isotonic-phosphate buffered saline pH 6.8 (buffer I). Serum albumin generally added to phosphate buffered saline as a stabilizing agent was omitted to avoid any possible uptake of sterols by this protein [19].

Toxoid. Streptolysin O was detoxified by adding a formaldehyde solution and was incubated at 37 °C as described elsewhere [4].

Assay of streptolysin O and toxoid. The hemolytic activity of previously reduced streptolysin was assayed with washed standardized rabbit erythrocyte suspension ($3 \cdot 10^8$ cells/ml) in buffer I as described previously [18]. One hemolytic unit is the amount of streptolysin O required to produce 50 % lysis of 0.5 ml of erythrocyte suspension after incubation at 37 °C for 45 min. It is equivalent to 2.7 ng ($2 \cdot 10^{-8}$ μM) protein [1].

The combining activity of the toxin with homologous antibodies was determined with standard anti-streptolysin O antiserum (Institut Pasteur Production, Paris). One combining unit of toxin (Lh T) is defined as the amount of streptolysin O which in the presence of one international unit of anti-streptolysin O still lyses 50 %

of erythrocyte suspension [18]. Under the experimental conditions employed, one combining unit of native active toxin is equivalent to 45 hemolytic units. The combining titer of streptolysin O toxoid, devoid of hemolytic activity, was determined by a blind-test technique [18].

Steroids. Cholesterol (5-cholesten-3β-ol), cholesterol acetate, cholestanone (5α-cholestan-3-one), coprostanol (5β-cholestan-3β-ol) were obtained from Steraloids (Pawling, N.Y., U.S.A.); lathosterol (7-cholesten-3β-ol), epicholesterol (5-cholesten-3α-ol) and cholenic acid (3β-hydroxychol-5-en-24 oic acid) from Schwarz-Mann (New York, N.Y., U.S.A.); dihydrocholesterol (5α-cholestan-3β-ol), 7-dehydrocholesterol (5, 7-cholestadien-3β-ol), β-sitosterol (24 ethyl-5-cholesten-3β-ol), 5α-cholestane, 20α hydroxycholesterol, estradiol-17β (1, 3, 5, (10) estratien-3, 17β-diol), 6-ketocholestanol (5α-cholestan-6-one-3β-ol) and lanosterol (8, 24 lanostadien-3β-ol) from Sigma (Saint-Louis, Mo., U.S.A.); 3 chlorocholestene (3 chlorocholesten-5), cholecalciferol (9, 10-seco, 5, 7, 10 (19)-cholestatrien-3β-ol), stigmasterol (5, 22, cholestadien-24-ethyl-3β-ol), tigogenin ((25 R)-5-spirostan-3β-ol), pregnenolone (3β-hydroxypregn-5-en-20-one) and dehydroepiandrosterone (3β-hydroxyandrost-5-en-17-one) from Merck (Darmstadt, G.F.R.); ergosterol (5, 7, 22-cholestatrien-24 methyl-3β-ol) from Fluka (Buchs, Switzerland); 11α-hydroxycholesterol (11α-hydroxy-5-cholesten 3β-ol) was a gift from Dr. Poirier (Roussel-Uclaf Laboratories) and α-ecdysone from Dr. Ozon (Paris VI University).

The sterols were checked for purity by thin-layer chromatography using different solvent systems [20]. A high degree of purity was found in sterol samples except for 7-dehydrocholesterol in which four spots were found.

Steroid aqueous suspensions. Steroids were dissolved in acetone (1 mg/ml) and then diluted in buffer I to the concentrations required. The dilutions obtained were dispersed by vigorous stirring in a Vortex apparatus (Scientific Industries, Springfield, Mass., U.S.A.) and used within 1 h. Dilute sterol-dispersions were dispensed by means of pipettes, rinsed several times with the dispersion owing to the extensive adsorption of sterols to glassware [21].

We also used cholesterol dispersion (50 μg/ml) in 0.01 % Tween 80 as recommended by Gershfeld et al. [21] and in lecithin-polyoxethylene glycol 6000 mixture as described by Badin and Denne [22]. The latter dispersion was kindly supplied by Dr. Badin (Hôpital Lariboisière, Paris).

Steroid agar gels. Glass slides (25 × 75 mm) were covered with 5 ml of melted agar gel prepared by incorporating 0.1–0.5 ml of steroid solution (1 mg/ml in methanol) under Vortex stirring into 5 ml of 1.5 % agar (Special Agar Noble, Difco, Detroit, Mich., U.S.A.) in NaCl solution (1.5 mg/ml). Diffusion and immunodiffusion of antigens alone or in the presence of antibodies solution were allowed to proceed from 5 mm holes punched in the gel (Figs. 2 and 3).

Immunodiffusion in tubes in the presence of steroids was performed as described in Fig. 4. Immunoelectrophoresis ran for 2 h under 20 volts with the same type of gels containing 0.1 M phosphate buffer pH 6.5 (Fig. 5).

Estimation of the inhibitory dose of sterol on hemolysis. Sterol dispersion suitably diluted was added to reduced toxin solution of known hemolytic titer according to the protocol of Fig. 1. After incubation at 20 °C for 30 min, the residual hemolytic titer of streptolysin O in the mixture was determined. The inhibitory dose 50 (ID_{50}) of sterol was calculated as described in Fig. 1.

Antistreptococcal antibodies. Concentrated human γ-globulins (16.5%), delivered by the Centre National de Transfusion Sanguine (Paris) having an antistreptolysin O titer of 600 I.U. per ml and giving a great number of precipitation lines with streptococcal exocellular proteins preparation [1], were used for immunodiffusion experiments. The term "antiserum" is used in the legends for γ-globulins.

RESULTS

Inhibition of the lytic effects of streptolysin O by cholesterol

The quantitative study of the inhibition of the lytic effect of streptolysin O on rabbit erythrocytes by increasing amounts of cholesterol (0.2 to 4 nmol) incubated with 100 hemolytic units of purified toxin preparation P_1 (270 ng equivalent to 4.5 pmol), has been carried out as described in Fig. 1. A non linear inhibition of lytic activity as a function of cholesterol concentration was observed for the three different aqueous dispersions of cholesterol used as well as for the other inhibitory sterols.

For this type of dose-response curve, the inhibitory dose 50 (ID_{50}) of cholesterol is more reliable than the 100% inhibitory dose, particularly for comparative purposes. For cholesterol dispersion in buffer I, the latter was around 40 ng (approx. 0.1 nmol) and the ID_{50} about 5 ng per hemolytic unit. At these concentrations cholesterol is soluble in the aqueous menstruum used, since the average solubility of this sterol in water or phosphate buffer is 1.8 ± 0.8 μg/ml corresponding to about $5 \cdot 10^{-6}$ M [21, 23].

In aqueous pseudo-solutions, cholesterol and related sterols occur in micellar states [23, 24] involving the stacking of the molecules which is concentration-dependent and greatly affected by the eventual presence of dispersing agents [19, 21]. Therefore, stacking is less pronounced in dilute cholesterol pseudo-solutions and the ID_{50} is likely to be a better quantitative parameter for the estimation of the inhibitory concentration of sterol; the enhancement of this effect has been reported when choles-

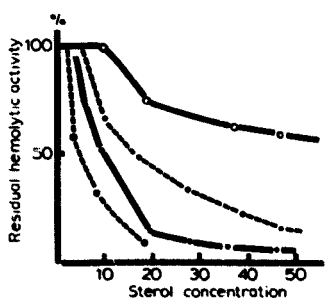

Fig. 1. Residual hemolytic activity versus sterol concentration. 10 ml of streptolysin O (10 hemolytic units/ml) were incubated at 22 °C for 30 min with 1 ml of sterol suspension. Residual hemolytic activity was determined as stated in Materials and Methods. The graph is plotted on the basis of the data recalculated for 1 hemolytic unit of streptolysin O. The ID_{50} of sterol is the quantity which decreases by 50% the hemolytic activity of the reduced toxin with respect to a control in which sterol dispersion is replaced by an equal volume of phosphate buffered saline (pH 6.8). (○—○), dihydrocholesterol; (●—●), 7-dehydrocholesterol; (●- - -●), cholesterol; (○- - -○), cholesterol, incorporated in lecithin-polyoxyethyleneglycol emulsion [22].

terol is incorporated with dispersing agents such as phospholipids [25], polyethylene glycol [22] or Tween 80 [19]. The potentiation observed is attributed to a better availability of cholesterol critical groupings to streptolysin molecules. We found a 3-fold increase of inhibition for the optically clear pseudo-solution of cholesterol dispersed in lecithin-polyoxyethylene glycol 6000 mixture (ID_{50} approx. 2 ng). In contrast no potentiation was observed with cholesterol dispersed in buffer containing 0.01 Tween 80 which has been reported to prevent the well-known extensive adsorption onto glass surfaces of cholesterol dispersed in buffer alone [21]. This may indicate that in our conditions even possible glass-adsorbed cholesterol molecules remain capable of combining with toxin as is the case for glass-adsorbed G_{M1} ganglioside towards cholera toxin [26]. It is to be noted that Tween 80 proved critical in cholesterol binding on *Acholeplasma laidlawii* membranes and polystyrene beads [21].

The stoichiometrical aspects of streptolysin O interaction with cholesterol will be discussed in another section of this article.

Structural requirements for inhibition of streptolysin O activity

Twenty four steroids differing by number and orientation of various polar or apolar groups, conformational state, side chain, number and position of double bonds have been tested. Only ten (Table I) proved inhibitory at various degrees. The structural requirements for inhibition were the following.

(1) *Presence of a 3β-(equatorial) hydroxy group on ring A of the cyclopentanoperhydrophenanthrene nucleus.* This strict stereochemical and chemical requirement

TABLE I

50% INHIBITORY DOSE OF VARIOUS STEROLS UPON THE HEMOLYTIC ACTIVITY OF STREPTOLYSIN O

Steroids were dispersed in phosphate buffered saline pH 6.8 from stock solution in acetone as described in Materials and Methods. The values of ID_{50} of the sterols employed have been determined by plotting residual activity of streptolysin O versus sterol concentration as illustrated in Fig. 1. Any steroid, the inhibitory effect of which was 50-fold less than that of cholesterol (250 ng per hemolytic unit), was considered as non inhibitory. One hemolytic unit of toxin is equivalent to 2.7 ng of protein. Non inhibitory sterols: epicholesterol, cholesteryl acetate, 3-chlorocholestene, dehydroepiandrosterone, pregnenolone, cholestane, 6-ketocholestanol, 5α-cholestane-3 one, lanosterol, estradiol, tigogenin, cholecalciferol, α-ecdysone.

Inhibitory sterols	Inhibitory dose 50	
	ng sterol/hemolytic unit	mol sterol/mol toxin
7 dehydrocholesterol	3	312
Cholesterol	5	520
Coprostanol	7	720
Dihydrocholesterol	10	1040
Sitosterol	10	1040
Lathosterol	10	1040
Stigmasterol	15	1560
Cholenic acid	20	2080
11 α-hydroxycholesterol	20	2080
20 α-hydroxycholesterol	50	5200
Ergosterol	50	5200

is illustrated by the ineffectiveness of any steroid having a modified 3β-OH group either by suppression (cholestane), oxidation (cholestanone), esterification (cholesterol acetate, 3-chlorocholestene) or epimerization into α position (epicholesterol with 3α-axial OH group)

(2) *Presence of a lateral aliphatic side chain of suitable size at carbon 17.* Suppression, shortening or lengthening of the hydrophobic isooctyl side chain of cholesterol or the introduction of functional groups on it greatly affect or suppress the inhibitory effect in the case of dehydroepiandrosterone which lacks side chain and tigonenin which has a complex cyclic structure instead of an aliphatic side chain. Modification of the eight-carbon chain by introduction of an ethyl group at C-24 (β-sitosterol) is not critical since only a 2-fold decrease of the inhibitory effect as compared to cholesterol is found. When the side chain has an extra double bond at C 22-23 and either $-C_2H_5$ (stigmasterol) or $-CH_3$ (ergosterol) group the inhibitory effect becomes 3-fold and 10-fold weaker. Watson and Kerr [8] reported that desmosterol (double bond at C 24-25) and fucosterol (= CH-CH_3 group) were as effective as cholesterol. With cholenic acid, which has a 5-carbon chain ending by a carboxy group at C-25, we observed a weak inhibitory effect as also shown by other authors who found that by contrast the methyl ester was inactive. Therefore, a non polar side chain appears to be not absolutely critical. Very poor inhibition was observed with 20 α-hydroxycholesterol which differs from cholesterol by a hydroxy group instead of a methyl group at C-20. Effective inhibition requires therefore the presence of this apolar group on the side chain.

(3) *Intact B ring.* This requirement is illustrated by the failure of cholecalciferol to inhibit toxin activity. This secosteroid which has both hydroxy group and isooctyl side chain has no longer the conformation of a steroid nucleus due to the rupture of B ring [27].

By contrast the saturated or unsaturated state of intact B ring and the positions of double bonds are not critical for inhibition. Dihydrocholesterol (saturated ring) and lathosterol (C-7, 8 double bond) are a little weaker than cholesterol, whereas 7-dehydrocholesterol (C-5, 6; 7, 8 double bond) was more inhibitory. On the other hand, the stereochemical relationships of rings A and B to each other are also not critical. The 5β *cis* (non planar) and the 5α *trans* (planar) steroids such as coprostanol and dihydrocholesterol respectively, originating from the presence of a chiral center at carbon 5 are both inhibitory. By contrast, only planar steroids inhibit polyene antibiotics [17].

The three above-mentioned criteria are minimal ones and have to be fulfilled simultaneously.

In general, any additional group either polar or apolar at various positions decreases or suppresses the inhibitory potency. Moreover, in a steroid molecule differing in several respects from cholesterol, no mutual compensation occurs between the different additional groups to create or enhance inhibitory effect. This seems the case for inhibitory steroids such as 6 ketocholestanol and lanosterol. The latter has two methyl groups at C-4 contiguous to the 3β-OH group.

Interaction of streptolysin O with sterols in gelified medium

(a) *Diffusion of streptolysin O in cholesterol-containing agar plates.* Highly purified preparation P_1 was allowed to diffuse from wells punched in agar plates into

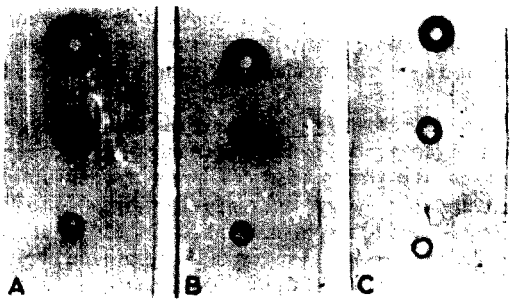

Fig. 2. Complex formation in agar gel between cholesterol and streptolysin O at various concentrations: A, B, C: 0.1, 0.2 and 0.5 ml of cholesterol solution in acetone (1 mg/ml), mixed with 5 ml of melted agar gel (50 °C). Wells from top to bottom, 10 μl of streptolysin O at respective concentrations of 3000, 1000 and 300 combining units/ml in buffer. Precipitation halos form overnight. After several washings with 0.3 M NaCl and distilled water, the plates were stained with Amidoblack acetic solution.

which cholesterol was incorporated at various concentrations (Fig. 2). Three different dilutions of toxin were introduced in each plate. Within a few hours, precipitation zones appeared progressively around toxin reservoirs. Diffusion was allowed to continue overnight. The plates were then repeatedly washed with 0.3 M NaCl and finally with distilled water to eliminate soluble materials or complexes and then stained with Amido Black. The halos appeared as blue discs visualizing the presence of streptolysin O as an insoluble material trapped in cholesterol matrix. Similar stainable halos were observed with other sterols of Table I which were found to inhibit toxin activity. By contrast, no halo formed with any of the non inhibitory sterols except for lanosterol which behaved in this respect as inhibitory sterols. We found the formation of similar halos when digitonin solution was allowed to diffuse similarly in cholesterol gels. This saponin is known to precipitate sterols which have a 3β-OH group by formation of insoluble complexes through binding with this group. It is very likely that like digitonin the halos which form between streptolysin O and inhibitory sterols are constituted by insoluble toxin-sterol complexes. The extent of complex formation is reflected by the variation of the area of precipitation halos depending on the relative concentrations of each reactant in the medium (Fig. 2). For a fixed concentration of cholesterol, we observed an increase of halo area with increasing concentrations of toxin or digitonin. By contrast, for a fixed concentration of these agents, the area of the complexes formed decreases for increasing concentrations of cholesterol which sequestrate them more strongly and reduce their diffusion owing to their insolubilization.

(b) *Double immunodiffusion of toxin against homologous antibodies in sterol-containing agar plates.* As shown in Fig. 3 for agar gels containing either cholesterol or epicholesterol, a normal antigen-antibodies immunoprecipitation band was formed between streptolysin O and anti-streptolysin O antibodies in epicholesterol gel whereas only a faint band occurred in cholesterol gel. By increasing cholesterol concentration in the gel, immunoprecipitation of toxin-antitoxin system was completely inhibited whereas normal reaction occurred under these conditions between diphteria toxin and

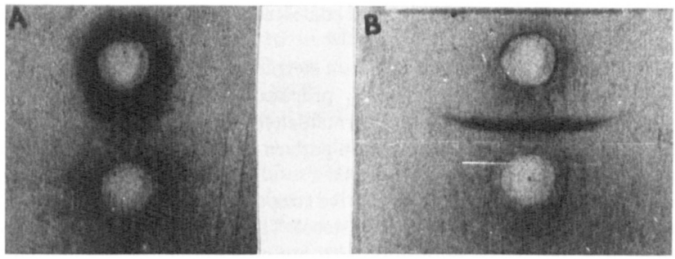

Fig. 3. Double immunodiffusion in sterol agar gel. A, cholesterol agar gel; B, epicholesterol agar gel. 0.5 ml of sterol solution in acetone (1 mg/ml) was mixed with 5 ml of melted agar (50 °C). Upper well, 10 µl of streptolysin O (300 combining units/ml) in buffer; bottom well, 10 µl antiserum (600 international combining units/ml).

homologous antitoxin taken as a control. By contrast to cholesterol, it is noted that no halo around toxin reservoir appeared in epicholesterol.

Therefore, streptolysin O is no longer able to react with precipitating homologous antibodies when complexed with inhibitory sterols. A similar finding was observed for neutralizing antibodies as reported below.

(c) *Specificity of streptolysin O affinity for sterols among streptococcal exocellular proteins.* Double immunodiffusion in cholesterol agar plate against human γ-globulins was performed with crude fraction (F_1) as described in Materials and Methods. The pattern of immunodiffusion bands in plain and cholesterol agar plates was similar except for a major band present in the former but not in the latter gel.

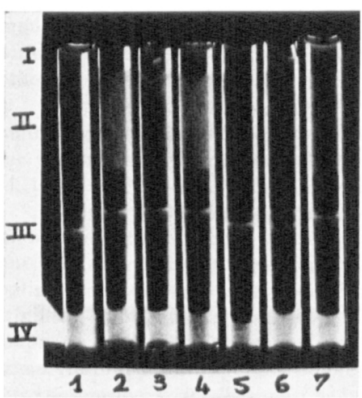

Fig. 4. Double immunodiffusion of streptolysin O and NAD glycohydrolase in sterol agar tubes. Four agar layers are disposed from top to bottom. (I), neutral agar onto which antigen solution is applied. (II), steroid agar, for streptolysin O trapping, prepared as described in Fig. 5. (III), neutral agar for immunoprecipitation reaction. (IV), antiserum incorporated in agar. (1), phosphate buffered saline; (2), cholesterol; (3), 7 dehydrocholesterol; (4), dihydrocholesterol; (5), dehydroepiandrosterone; (6), cholestane; (7), lanosterol.

We checked in a control experiment that this band coalesced with that of pure streptolysin O placed in the same plate.

We also observed that only native toxin has a steroid binding capacity. Immunologically reactive but hemolytically inactive F_1 preparation modified by thermal denaturation at 45 °C or by action of 0.1 % formaldehyde (toxoid) did not form halo in cholesterol gel and gave an immunodiffusion pattern identical to that of control gel.

These experiments show that except for native streptolysin O the other streptococcal exoproteins do not exhibit affinity for cholesterol. This is especially the case for NAD-glycohydrolase. The purified preparation (P_2) containing both enzyme and toxin was allowed to diffuse in agar tubes against γ-globulins through a gel-layer containing sterol. Two immunodiffusion bands were formed in control plain agar tubes and in those containing any of the non inhibitory sterols except lanosterol (Fig. 4). The latter and inhibitory sterols retain streptolysin O in the sterol layer acting as a microchromatography affinity column since, only one diffusion band is formed corresponding to the NAD glycohydrolase-antibody system. In this case lanosterol also exhibits a particular behavior as it seems to be complexed by streptolysin O without inhibition of its lytic properties.

(d) *Immunoelectrophoretic analysis of streptolysin O and NAD-glycohydrolase in cholesterol agar gel.* As streptolysin O is specifically complexed by surrounding cholesterol molecules, it appeared interesting to investigate toxin behavior under an electric field in cholesterol agar gel as compared to NAD-glycohydrolase which has no affinity for this sterol. Immunoelectrophoresis of P_2 preparation was carried out in normal and cholesterol agar gel (Fig. 5). In the latter only NAD-glycohydrolase migrates and gives a precipitation arc with human γ-globulins whereas streptolysin O remains trapped around antigen reservoir. Therefore, streptolysin O-cholesterol complex is neither dissociated nor displaced under an electric field. In a control experiment carried out in epicholesterol agar gel both antigens migrate normally as in plain agar and form corresponding immunoprecipitation arcs.

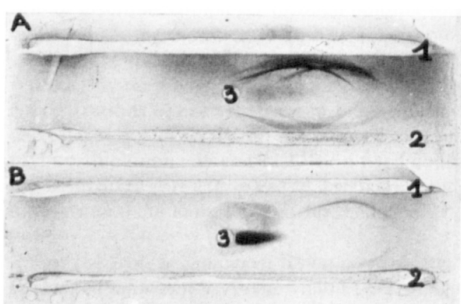

Fig. 5. Immunoelectrophoresis of streptolysin O and NAD-glycohydrolase. (A), neutral agar; (B), cholesterol agar gel as described in Fig. 5. (1), antiserum (600 international combining units/ml); (2), three-fold diluted antiserum; (3), antigen: 10 μl of partially purified streptococcal preparation containing streptolysin O and NAD glycohydrolase obtained by isoelectric focusing [1]. Immunoelectrophoresis was performed in 0.1 M phosphate buffered saline pH 6.5 for 2 h at 22 °C (20 volts).

Effect of sterols on toxin neutralization by antistreptolysin O antibodies

After incubation of toxin with inhibitory or non-inhibitory sterols, the combining titer of streptolysin O with neutralizing antisera was determined as for a toxoid : blind-test technique [18].

Preparation P_1, diluted to 2 combining units/ml, was incubated at 20 °C for 15 min with inhibitory sterol added at the limit concentration giving full inhibition of the lytic activity. Non inhibitory sterols were added at equivalent molar concentrations. At the end of the incubation time an equal volume of active toxin dilution at the same concentration was introduced in each toxin-sterol mixture and the overall combining titer was determined with titrated antisera. We used human γ-globulins, anti-streptolysin myeloma and various equine antisera as previously described [28]. The neutralizing antibodies are either directed against fixation site or lytic site(s) or both [4]. For all sera or antibodies tested, we observed that for toxins incubated with inhibitory sterols the titer found was that of the additional toxin added after incubation with sterol whereas, for non-inhibitory sterols including lanosterol or sterol-free controls, toxin titer was twice as high (overall titer). This also shows that streptolysin O molecules already complexed with cholesterol are no longer able to combine with any type of homologous neutralizing antibodies.

DISCUSSION

Streptolysin O appears as a protein which specifically binds cholesterol and other sterol molecules possessing a 3β-OH group and aliphatic side chain provided that other groups do not occur near these two critical and stereospecific structures to prevent interaction. This steroid-binding property is unique among bacterial protein toxins and similar to that known for a number of proteins from higher organisms such as cellular receptors of steroid hormones or various serum proteins which specifically bind and transport steroids. Strict antipodal structural or conformational specificities of steroids are required for binding as described in this article. It is also the case for the other proteins mentioned above [29, 30].

Upon steroid binding, streptolysin O loses its lethal properties on laboratory animals and its cytolytic effect on eukaryote cells, most likely by competitive inhibition for its binding on membrane cholesterol which is required for triggering cytolysis [4, 13].

Highly purified streptolysin O, free from other streptococcal proteins, was used for a quantitative and comparative estimation of inhibitory potency of sterols and as a possible approach to the stoichiometry of streptolysin O-sterol interaction. Under optimal conditions of cholesterol dispersion one hemolytic unit of toxin (2.7 ng) was inhibited by about 4 ng of cholesterol. This value is close to that reported for crude preparations by Delattre et al. [25]. We found similar values for inhibition of one hemolytic unit of partially purified preparations of tetanolysin, *Clostridium perfringens* θ-toxin, botulysin, *Bacillus alvei* hemolysin and listeriolysin (unpublished) immunologically and chemically related to streptolysin O [13].

Similar values of inhibition by cholesterol have been reported for pneumolysin [31] and θ-toxin [32]. The much higher inhibitory amounts of cholesterol (0.14–1.5 μg) reported by others remain unexplained [8, 33].

We have also shown that the immunochemical properties of streptolysin O

are also modified, since toxin mixed to cholesterol no longer combines with neutralizing and precipitating antibodies. Kaplan and Wannamaker [10] reported that the immunogenicity of the toxin is greatly reduced or suppressed as evidenced by the significant depression in antibody response in rabbits injected with a mixture of streptolysin O and cholesterol-containing skin lipids. Among other biological properties affected by cholesterol one should mention the suppression of the inhibitory effect of sublytic doses of streptolysin O and θ-toxin on chemotaxis and mobility of neutrophilic leukocytes [34, 35] and the stimulation of human lymphocyte transformation in vitro [36]. We established that insoluble complexes form in vitro between inhibitory sterols and streptolysin O, whereas non inhibitory sterols except lanosterol do not react. The deviating behavior of this sterol awaits further investigation.

The complexes formed were visualized by allowing the diffusion of streptolysin O in gelified medium containing any inhibitory sterol. A similar precipitation pattern was found under the same conditions with digitonin. Owing to the strict stereospecificity of inhibition or insolubilization, complex formation appears as a specific sterol-protein interaction. Holmgren et al. [37] also observed in agar gel the formation of an insoluble precipitation band upon diffusion of cholera toxin against GM_1 ganglioside. This ligand specifically inactivates the toxin in vitro and is known to be its cellular receptor.

Our findings are supported by the experiments of Duncan and Schlegel [38] on streptolysin O and those of Smyth et al. [39] on θ-toxin who observed by electron microscopy, ring and arc-shaped structures when these toxins are mixed with cholesterol dispersions or after interaction with natural or artificial cholesterol-containing membranes. These structures did not appear with the non inhibitory cholesterol esters. A hypothetical model of conformational change in the streptolysin O molecule has been proposed by Duncan and Schlegel [38].

The stoichiometry of streptolysin O-cholesterol interaction in vitro was not possible to estimate accurately. As cholesterol occurs in complex polymorphic micelles in water [23, 24] only one or few molecules of micelles are likely to interact with the specific cholesterol binding site(s) on any molecule of toxin. In addition, it is difficult in this case to interpret any stoichiometric data since binding of any amphiphilic ligand to a protein involves competition with the self association of the amphiphile [40].

The ratio of 500 molecules of cholesterol to inactivate one molecule of toxin calculated from the data based on the evaluation of the inhibitory dose 50 per hemolytic unit (Fig. 1) is but a rough figure of the actual ratio. It is to be noted that the ratio of ganglioside GM_1 to deactivate cholera toxin is 300 to 1 [41].

The formation of hydrophobic complexes in strict stoichiometric ratios has been reported for polyene antibiotics [15, 17] and some saponins [14] which like streptolysin O and related toxins are cytolytic. Their activity is inhibited by those sterols responding to the same structural features found for these toxins except for the non-planar (5β *cis*) steroid nucleus. In spite of the very different structures between these agents a close similarity occurs as regards their interaction with sterols in vitro and with membrane cholesterol in vivo as evidenced by biological propertie and electron-microscopic pattern [12, 15].

Cholesterol is a critical constituent of the biomembranes of eukaryote cells involved in the regulation of the rigidity, stability and permeability of the bilayer

through a potent condensing effect on phospholipids [42, 43].

It is to be noted that the very sterols shown to inhibit streptolysin O or polyene activity have been found to ensure maximum stability in model artificial systems or in biomembranes in which cholesterol was replaced by these sterols [44]. The high affinity of streptolysin O or polyenes to cholesterol most likely deviates this sterol from its interaction with phospholipids and considerably weakens membrane stability leading to cell collapse. The molecular mechanism and the regions of the protein molecule by which streptolysin O interacts with cholesterol and related sterols in vitro and in vivo as well as the nature of the bonds involved and the composition of the complexes still remain to be established. It is to be noted that according to the presently accepted models of biomembranes [24, 42] only the 3β-OH group of this sterol is exposed to the water interface at cell surface, whereas the side chain extends towards the hydrophobic center of the bilayer and is therefore not available for toxin binding. One may thus speculate why this side chain is required in addition to the OH group in vitro for sterol-toxin interaction and the Centre National de la Recherche Scientfique.

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