

Effect of *Staphylococcus* Toxins Isolated from Dairy Cow Milk on Vero Cell Monolayers

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ABSTRACT. The effect produced on Vero cell monolayers by toxins derived from *Staphylococcus* strains was characterized. 210 milk samples taken from dairy cows suffering from sub-clinical mastitis were analyzed. Strains belonging to the *Staphylococcus* genus were isolated from 73 of these milk samples. The production of toxins was then stimulated from these strains when they were cultured in Dolman's medium. The study of cell cultures showed that 53 toxin samples induced marked and irreversible cellular changes. This is compared to 42 samples (57.5%) which were strongly cytotoxic. The remaining 11 samples were shown to be slowly cytotoxic. 16% of the total toxins did not induce cell damage and 11% of the toxins produced cellular damage that was reversible in less than 24 hrs, and were designated as cytotoxic. Haemolytic activity *in vitro*, using sheep red blood cells, was assessed using toxins that caused alteration in the monolayers. The results indicate that 46.51% of the toxins showed **b** haemolytic activity, 2.32% **a** haemolytic activity, and 51.16% showed neither **a** nor **b** haemolytic activity. The later type of activity did however cause damage to cultured cells, which suggests that the causative agent could be **d** toxin. This study reveals a strong predominance of **b** haemolytic strains in the dairy farm studied. These strains induced *in vitro* cell damage, and it is possible to speculate that mammary gland tissue damage is similarly produced, which may be attributed to both **b** and/or **d** haemolytic toxins.

Key words: *Staphylococcus aureus*, Cytolysins, Vero cell cultures, Haemolytic activity, Bovine mastitis.

RESUMEN. El efecto de toxinas de cepas de *Staphylococcus* sobre monocapas de células Vero fue caracterizado. 210 muestras de leche tomadas de vacas afectadas de mastitis subclínica fueron analizadas. De esas muestras fueron aisladas 73 cepas pertenecientes al género *Staphylococcus*. La producción de toxinas por las cepas fue estimulada cuando se cultivaron en medio Dolman. El estudio realizado en cultivos celulares demostró que 53 muestras de toxina indujeron cambios celulares marcados e irreversibles. Esto es comparado a 42 muestras (57.5%) que fueron fuertemente citotóxicas. Las 11 muestras restantes mostraron ser ligeramente citotóxicas. El 16% del total de las toxinas no indujo daño a las células y 11% de las toxinas produjeron un daño celular, designado como citotónico, que fue reversible dentro de 24 h. La actividad hemolítica *in vitro* sobre eritrocitos de borrego fue probada usando toxinas que causaron alguna alteración sobre las monocapas. Los resultados indican que el 46.51% de las toxinas presentaron una actividad **b** hemolítica, 2.32% actividad **a** hemolítica, y 51.16% no mostraron actividades tipo **a** o **b**. Esta última actividad dañó las células posiblemente por una toxina **d**. Este estudio revela una fuerte predominancia de cepas **b** hemolíticas en la granja lechera estudiada. Esas cepas indujeron daño *in vitro*, y es posible especular que el daño en el tejido de la glándula mamaria puede ser atribuido a las toxinas hemolíticas **b** y **d**.

Palabras clave. *Staphylococcus aureus*, Citolisinas, Células Vero, Actividad Hemolítica, Mastitis Bovina.

INTRODUCTION

Studies on the etiology of intra mammary bacterial infections have shown that 95% of such infections are caused by the *Staphylococcus* and *Streptococcus* genera.⁵ The most isolated bacterial species being *Staphylococcus aureus*.^{6,7}

In an attempt to evaluate and characterize the *Staphylo-*

coccus genus it is necessary to be aware of certain important epidemiological indicators, such as the frequency of presentation and prevalence of the microorganism, risk factors, pathogenicity and virulence factors.⁶ These indicators may then be applied to the analysis of bovine intra mammary infection dynamics.

Toxins derived from species belonging to the *Staphylococcus* genus make up some of the virulence factors that



contribute to the development of the clinical picture. Amongst these toxins are found the haemolysins, which have been denominated as α , β , γ and δ .^{2,17} The haemolysins are differentiated by their different activities manifested on erythrocytes from different animal species, although they can also produce toxic effects on other cell types such as, fibroblasts, macrophages and white blood cells.⁵

The haemolysins can be detected by immunological tests or in certain animal models, and when released into culture medium may be analyzed according to the effects they induce on cell cultures. The work presented here focuses on the characterization of *Staphylococcus* strains isolated from milk taken from cows with sub-clinical mastitis. Their toxigenicity on Vero cell cultures has been assessed, and in parallel this phenotypic expression has been compared with *in vitro* haemolytic activity. It is suggested that this *in vitro* model could then be used to simulate tissue damage found the *in vivo* situation.

MATERIAL AND METHODS

Samples. During September 1995 to August 1996, milk samples were taken from a dairy farm situated 12 Km from Río Cuarto city, Córdoba, Argentina. A total of 210 milk samples were taken manually from different cow teats, and were collected into sterile containers. Care was taken to maintain the greatest aseptic conditions possible, and the samples were kept cold until they were later processed in the laboratory.

Bacteriological examination. Each milk sample was placed in Hotis medium (0.5 ml 0.5% purple bromocresol + 9.5 ml milk).¹⁴ The cultures were then incubated at 37°C for 24 h. Following this initial incubation, colonies were isolated by streaking drops onto Petri dishes containing manitol agar salt (Merck) and were incubated for 24 h at 37°C.

The colonies were then stained with Gram stain, and those cultures whose cocci were both Gram positive and positive for the catalase test were selected.³ To select strains belonging to the *Staphylococcus* genus, the following tests were carried out: Resistance to bacitracin (at 0.04 U/ml),⁸ fermentation of glucose and motility.¹⁹ In addition, the rapid coagulase binding test was carried out.¹⁰ In order to select for haemolytic strains, those strains identified as belonging to the *Staphylococcus* genus were seeded into Petri dishes containing sheep tryptose blood agar (5%).

Toxin production. The selected strains were seeded in brain heart broth and incubated for 12 h at 37°C. In order to induce production of the *Staphylococcus* toxins 1 ml was taken from these cultures and placed in semisolid Dolman medium. The cultures were then incubated at 37°C for 24 h with 20% CO₂.²¹ Cultures were later filtered through Whatman N°2 paper, and centrifuged at 8,000 rpm. for 15 min at 4°C.⁹ The supernatants were then sterilized by filtration through 0.22 μ m Millipore filters. The control for sterility

was carried out in thioglycolate medium.

Toxigenicity test in cell cultures. The Vero cell line used for this assay, was obtained from the Argentine Cell Bank Association (ABAC), and had undergone 40-72 passages. Cell monolayers were grown in 96 well micro-plates with Eagle's minimal essential medium (MEM), plus Earle's salts supplemented with 8% fetal bovine serum.¹³ To these cell cultures either the undiluted supernatants were added, or supernatants diluted by a factor of 2 or 5, in maintenance medium (MM) consisting of MEM supplemented with 2% fetal bovine serum. The assays were carried out in triplicate and were incubated for no more than 7 days at 37°C. The control consisted of cell monolayers containing MM alone.^{9,11}

Microhaemagglutination test. Bacterial culture supernatants that were found to induce cellular change in the monolayers, were selected and submitted to analysis of haemolytic activity. This was carried out by adding a 1% suspension of sheep red blood cells in sterile physiological solution to each supernatant diluted by a factor of 2. This mixture was then incubated in 96 well "U" bottomed micro-plates for 1 hour at 37°C. At this temperature it was possible to determine α -haemolysis activity. The micro-plates were later incubated at 4°C over night (*hot-cold shock*), which revealed the β -haemolysis activity.¹

RESULTS

Bacteriological analysis. Of the total 210 milk samples collected and cultured on manitol salt agar medium, it was possible to select 73 strains which had clusters of Gram positive cocci. 100% (73) of these cultures were also found to be positive to the catalase test.

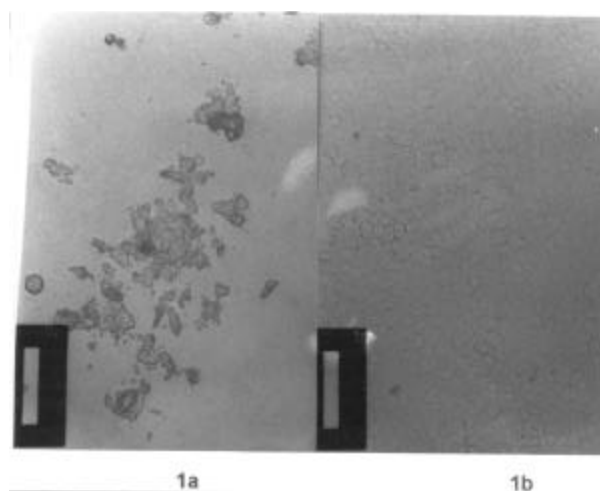


Fig. 1. a) Fast cytotoxic effect of *Staphylococcus* toxins (strongly cytotoxic or SCT) on Vero cell monolayer cultures. Cell damage was observed before 24 h p.i. b) Cytotonic (CT) effect of staphylococci toxins induced on Vero cell monolayers, before 24 h p.i. (X 20).

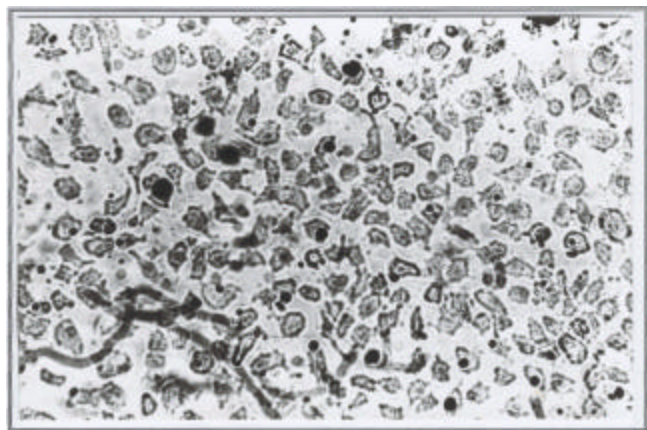


Fig. 2. Cytotoxic effect of *Staphylococcus* toxins, induced on Vero cell monolayers at 10-12 hours p.i. (X 40). These toxins were characterized as being slowly cytotoxic toxins or SCTb.

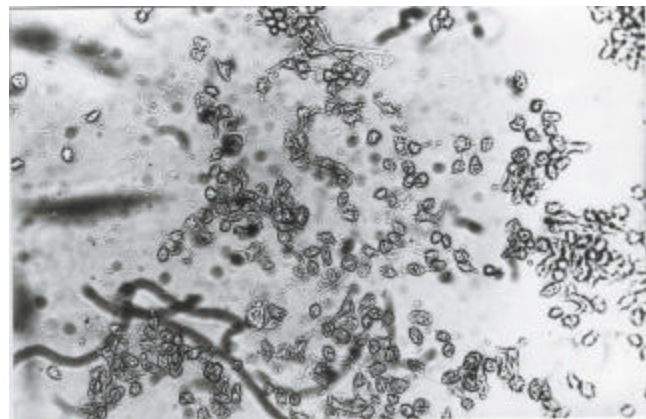


Fig. 4. Cytotoxic effect of *Staphylococcus* toxins on Vero cell monolayers at 36 hours p.i. These toxins were characterized as slowly cytotoxic toxins or SCTb (X 40).

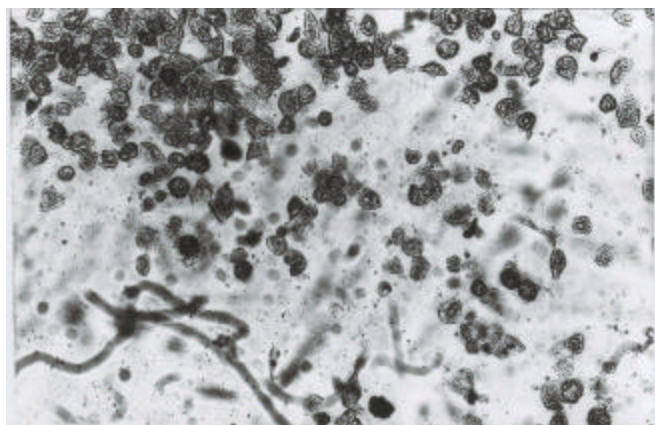


Fig. 3. Cytotoxic effect of *Staphylococcus* toxins, induced on Vero cell monolayers at 24 hours p.i. These toxins were characterized as slowly cytotoxic toxins SCTb (X 40).

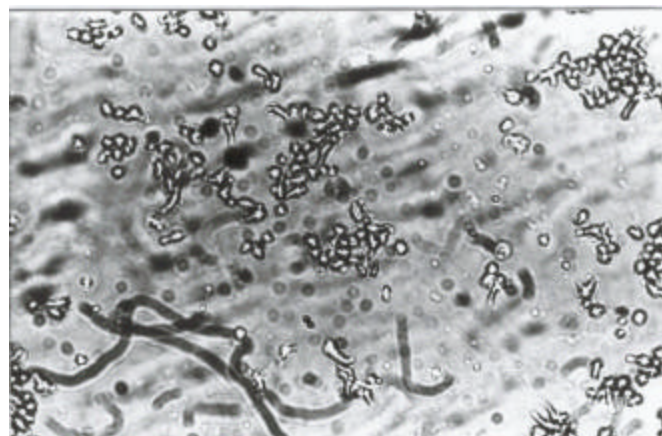


Fig. 5. Cytotoxic effect of *Staphylococcus* toxins on Vero cell monolayers at 48 hours p.i. These toxins were characterized as slowly cytotoxic toxins SCTb (X 40).

All strains tested were resistant to bacitracin, fermented glucose and were non-motile. Based on these criteria they were therefore considered to be members of the *Staphylococcus* genus.¹⁸

Toxicity test in Vero cell cultures. Cell damage induced by the supernatants was analyzed by inverted light microscopy. The effect of each supernatant was characterized according to the following four definitions:

a) *Strongly cytotoxic toxins (SCT)*: This category included those samples that caused strong cellular destruction, which was evident and irreversible during 24 h post inoculation (p.i.), (Fig. 1a). The number of strains that induced toxins with these characteristics was 42 (Fig. 7).

b) *Slowly cytotoxic toxins (SCTb)*: This category includes those samples that caused irreversible cellular death, in a progressive manner over time, not exceeding 7 days p.i., (Fig. 2, 3, 4 and 5). The number of strains that induced toxins with these characteristics was 11 (Fig. 7).

c) *Cytotoxic toxins (CT)*: This category includes those samples that induce rounding up of cells, with reversion to the normal state in no less than 24 h p.i., (Fig. 1b). The number of strains that induced toxins with these characteristics was 8 (Fig. 7).

d) *Negative toxins (NT)*: This category includes those samples that did not induce changes in the cell monolayers, and which were found to be identical to the untreated controls, (Fig. 6). The number of strains with these characteristics was 12 (Fig. 7).

Microhaemagglutination test. The analysis of haemolytic activity was carried out on the 43 strains that were found to produce SCT and SCT^b effects on Vero cells.

The results of the haemolytic activity on sheep red blood cells are shown in Table 1. It was possible to detect one strain with a pure α -haemolysis effect (2.32%). This is compared with the high activity of β -haemolytic strains (18) (41.86%), and two strains with α - β haemolysis activ-

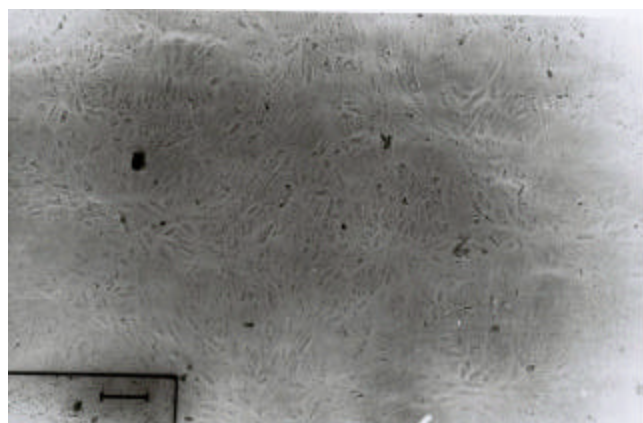


Fig. 6. Control Vero cell monolayer cultured without toxins (X 20).

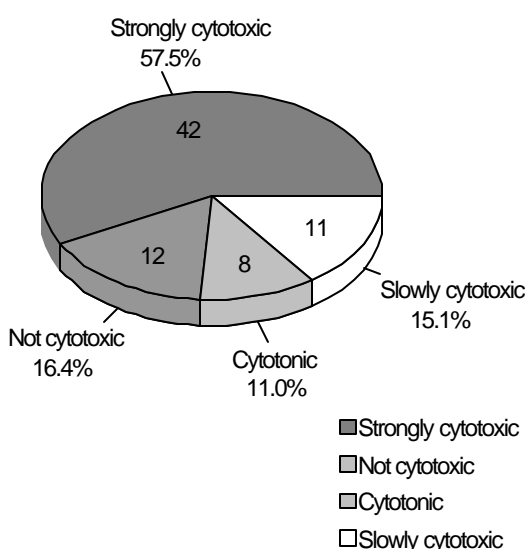


Fig. 7. Classification of the effects of *Staphylococcus* toxins on Vero cell monolayer cultures

ity (4.85%), whose joint activity added to the activity of both toxins (α and β) increased the value to 48.83% (21). As an example, Fig. 8 shows the results of the haemolytic activities produced by the same toxins when the test was developed at 37°C and then submitted to *hot-cold shock* (Fig. 9). The supernatants of the remaining 51.56% (22) strains did not show α and/or β haemolysis activity.

DISCUSSION

The 73 strains belonging to the *Staphylococcus* genus were analyzed according to their capacity to generate

Table 1. Classification of the haemolytic activity of staphylococcus toxins by the micro-haemoagglutination test using sheep red blood cells.

Type of haemolysin	Positive	Haemolytic titre (range)	Percentage	Characterized in CC•
α	1	1/2-1/4	2.32	SCT
β	18	1/2-1/32	41.86	SCT
α and β	2	1/2-1/32	4.65	SCT
No α -No β	11			SCT
No α -No β	11		51.16	SCTb
TOTAL	43	-	100	SCT

CC•: Cell culture; SCT: Strongly Cytotoxic; SCTb: Slowly cytotoxic (there were 3 in total).

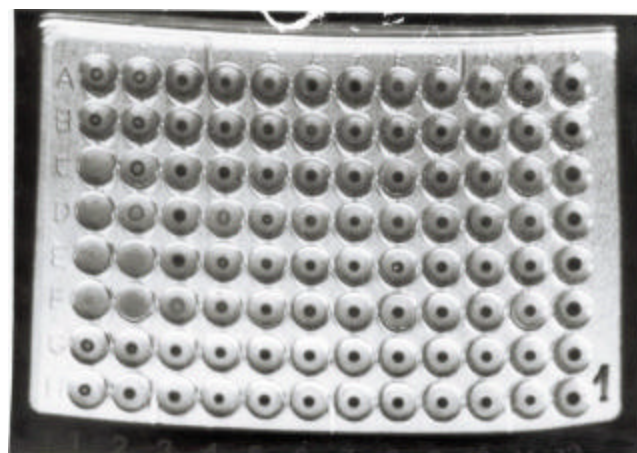


Fig. 8. Haemolytic effect of staphylococcus toxins on sheep red blood cells, carried out at 37°C.

changes in *in vitro* cell culture systems. The Vero cells, set up as a cell line, are mammalian cells, which implies that the results obtained may reflect the activity of toxins in the mammary gland *in vivo*.

In support of this there is information in the literature on

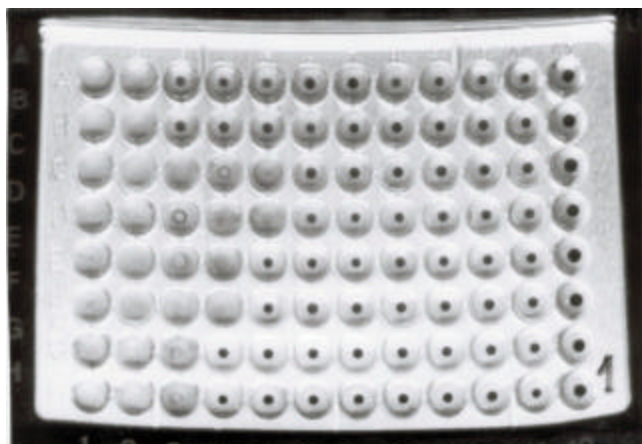


Fig. 9. Haemolytic effect of *Staphylococcus* toxins on sheep red blood cells when "hot-cold shock" was applied.

toxins derived from both the *Staphylococcus* genus,^{5,20,21} and from other bacteria.^{11,9,15,16} Based on the definition given by these authors it was also possible to define toxins within the samples studied, as cytotoxic or cytolytic. According to their criteria it was also possible to identify toxins that induced progressive and irreversible rounding up of cells, ending in lysis or death with total detachment of the cell monolayer. According to our observations it is also possible to distinguish two sub-categories of toxins within this category, as based on the speed at which the cells died. Such that, when cell death occurred between 18 and 24 h p. i. the cells were characterized as being highly cytotoxic, and those designated as slowly cytotoxic, totally destroyed the monolayers at 7 days p.i. These two sub-categories suggest that the strains that produced them are different with respect to this virulence factor, since the character of the strain producing the toxin is an intrinsic factor for each species.

It is necessary to point out that although the production of toxins in all the mono-microbial cultures is induced in Dolman's medium under identical laboratory conditions, the level of toxin production would not be the same for each of the isolated species or perhaps the mechanisms of action would differ. Amongst the toxins produced by the staphylococci the haemolysins are the most prevalent. Hence, in the light of the fact that these toxins produce a marked lytic action on erythrocytes from different animal species as well as on other cell types, we infer that the activity described in our assays as cytotoxic, may be due to said haemolysins. This hypothesis was supported by the correlated study of the haemolytic activity on sheep red blood cells.

It is known that β toxin is a phospholipase that provokes hydrolysis of the sphingomyelin component of cell membrane phospholipid bilayers. This toxin does not induce cell lysis but does generate instability of cell membranes, making cells susceptible to the action of certain effects such as, marked temperature variation, and changes in pH

and ionic strength.¹⁷ Taking this into account the action of temperature is a relevant parameter that permits the identification of β toxin when erythrocytes are submitted to hot-cold shock.

When the haemolytic assay was carried out at 37°C, the sheep red blood cells were altered by the effect of α and β toxins. It is known that at this temperature α toxin acts to induce lysis of the cells, such that it is possible for it to be detected and titred (Fig. 7). However, at this temperature, although β toxin makes the erythrocytes extremely fragile, its haemolytic activity is only evident following exposure to 4°C (Fig. 8).

With these findings it was possible to analyze the results obtained from the development of the haemoagglutination technique, which are summarized in Table 1. It can be seen that a greater percentage (51.16%) did not show haemolytic activity. In contrast, amongst those that did reveal this activity, there is a large percentage (41.86%) that could be characterized as having β haemolytic activity, and when the joint effect of α and β haemolytic activity is accounted for there is an increase to 48.83%. A low percentage (2.32%) were characterized as being due to pure α haemolysis.

The comparative analysis of the α and β haemolysis values shows a marked incidence of strains producing β cytolysins. These results are not surprising when the epidemiological aspects that characterize the dairy farm used in the study are taken into consideration, since preliminary assays carried out indicated that approximately 40% of the strains produce this cytolysin.⁷

Furthermore, some authors have stated that the prevalence of cytolytic β strains is characteristic of staphylococcus cocci isolated from the bovine mammary gland. This indicates the adaptation of these strains where the predominant target of toxin activity is sphingomyelin.^{5,23}

The maintenance in time of the same epidemiological pattern indicates that the assays carried out have been consistent. In addition, the assays help towards the use of new methods for dairy farm management and preventative strategies, when strains involved in the pathology as well as their virulence factors, have been adequately identified.

In contrast, the finding that there is a lower incidence of strains producing α haemolysis (2.32%) is justified by epidemiological aspects, are further supported by the literature that cites that strains with these characteristics are more prevalent in human staphylococcal infections.⁵

The cytotoxic effect of β cytolysin has also been described in cell cultures of different origins, such as cells lines set up with HeLa cells, fibroblasts and human thrombocytes, etc. However, there are contradictory results of cytotoxic effect for different cell models, such that effects could not be observed when the inoculation medium of the toxin lacked Mg^{++} ions.^{21,22} This limitation did not exist in our assays, which indicates that the observed cytotoxicity can be attributed to the individual and/or joint activity of α



and β cytolysins. On the other hand, it's worth pointing out that all the toxins with haemolytic activity, shown in table 1, were characterized using strongly cytotoxic cell cultures. It has been stated in the literature that the combination of α and β toxins strengthens their activity and induces cell damage that is rapidly marked.⁵

Table 1 also shows that the higher percentage of toxins with cytotoxic activity in cell cultures did not however show haemolytic activities (51.16%). It is possible to speculate that damage caused to the cell monolayers is due to another type of toxin produced by other species of the genus. Many haemolytic toxin preparations are often contaminated with other toxins, which cause membrane damage. In particular the δ toxin that is known to cause lysis of bacterial protoplasts, breakage of mitochondrial membranes, and inhibition of ATPase activity dependent on Na^+ and K^+ , all of which are not properties of the cytolysins analyzed here.¹²

Rodríguez-Angeles *et al.* analyzed activity induced by the cholera toxin on Vero cells, and defined cytotoxic activity as that which produces reversible cellular lengthening starting after 24 h. A similar definition was given by Giono-Cerezo *et al.* who studied *E. coli* cytotoxins in the same cell system.

In the same way as these researchers, our results reveal cellular alteration which is reversible to the normal condition during the course of 24 h p.i. However, more than lengthening, it was possible to visualize clear enlargement of the cells with polyhedron forms, which was made easier by the confluence of the cell monolayer (Fig. 1b).

It should be pointed out that amongst the toxins produced by Staphylococci, the enterotoxins have been described (A, B, C, D, G and H).⁶ It is possible that their production was produced when the strains were replicated in the Dolman's medium. However, although the mechanism of activity of these Staphylococci enterotoxins has not been expressed in the cell systems, it cannot be discounted that they were the cause of the observed damage.

In support of this hypothesis Giono-Cerezo *et al.* described cytotoxic activity produced by the *E. coli* thermolabile (TL) enterotoxin using Vero cells.

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