The hemolytic effect of *Salmonella typhi* Ty 2 porins

Inés CALDERON, Sergio R. LOBOS, and Guido C. MORA

Laboratorio de Microbiologia e Inmunologia, Facultad de Ciencias Biológicas, Pontificia Universidad Católica de Chile, Santiago

Two outer membrane proteins of *Salmonella typhi* Ty 2 were extensively co-purified. According to their migration in dodecylsulfate/polyacrylamide gel electrophoresis and solubility characteristics, these proteins are homologous to the 35-kDa and 36-kDa porins found in *Salmonella typhimurium*. A porin homologous to the 34-kDa one has not been found in *S. typhi* Ty 2. A critical step in the purification of porins is heating at 100 °C in 2% sodium dodecyl sulfate before Sephadex gel filtration. The absence of detergent in aqueous suspensions enhances porin aggregation, these aggregations inducing human red cell lysis. Porins obtained by an alternative procedure consisting of heating at 60 °C instead of 100 °C were also hemolytic. Using nanomolar concentration of porins a strong influence of temperature on the hemolytic effect was observed. Porin-induced hemolysis was inhibited with anti-porin serum, as well as by a treatment with phenylglyoxal, which reacts with the arginine residues of proteins. The membrane-disrupting ability of porins aggregates might explain some pathogenic characteristics of gram-negative bacterial infections.

Outer-membrane surface components of gram-negative bacteria have been shown to be deeply involved in the interactions with the host cells during infection [1–4]. Adherence to epithelial cells followed by penetration and invasion of the host systemic circulation are likely to be mediated by outer-membrane components of some gram-negative bacteria, although periplasmic enzymes might also contribute to the invasiveness of bacteria [5].

Fluidity changes and increased hydrophobicity of epithelial cells are induced by bacterial surface components [5] and it is possible that outer-membrane proteins may also alter the permeability of the host cells. It has been shown that *Escherichia coli*, *Salmonella typhimurium* and *Proteus mirabilis* porins [6–8] are capable of forming transmembrane permeability channels in artificial vesicle membranes and that this capability is dependent upon the formation of porin oligomers [7,9]. This property resembles the behaviour of alamethicin [10], and other related peptide antibiotics that have channel-forming ability [11–14]. As a result of this ability, alamethicin will also induce human red cell lysis [15], leukocyte lysis [16], increased calcium permeability of sarcoplasmic reticulum vesicles [17], uncovering of latent adenylate cyclase activity [18] and fusion of lipid vesicles [19].

It was of interest to determine whether porins also show a capacity to induce lysis of human erythrocytes, since this may help to understand some of the events occurring as a result of infection with pathogenic gram-negative bacteria. In fact, hemolytic anemia has been found in some typhoid fever patients, specially in those with glucose-6-phosphate dehydrogenase deficiency [20]. We have extensively purified *Salmonella typhi* Ty 2 porins and tested their capacity to induce human erythrocytes lysis.

Abbreviations. SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

Bacterial strains

Salmonella typhi Ty 2 was a kind gift from the Instituto de Salud Pública de Chile. *Salmonella typhimurium* was from our strain collection.

Isolation of S. typhi *Ty 2 porins*

Bacteria were grown in Carlquist ninhydrin base medium (Fisher Scientific) with 1.5% agar with which approximately 25 g (wet weight) of cells were harvested. Outer membranes were obtained by the procedure of Moore et al. [21]. Briefly, the membrane pellet obtained after sonication of cells was suspended in 10 mM Tris/MgCl buffer, pH 7.8, containing 2% Triton X-100 and centrifuged until a Triton-insoluble pellet consisting of outer-membrane proteins was obtained.

The outer-membrane preparation resuspended in 10 mM Tris/HCl buffer, pH 8.0, containing 10 mM $MgCl_2$ and 5 mM NaN_3 was treated with lysozyme (100 μg/ml) and incubated at 37 °C for 2 h. The pellet obtained after centrifugation at 90000 × *g* for 60 min was resuspended in 10 mM Tris/HCl buffer, pH 7.8 containing 10 mM $MgCl_2$, 2% SDS and 0.05% 2-mercaptoethanol, heated at 100 °C for 5 min and passed through a Sephadex G-150-120 column (50 × 1.8 cm). An alternative procedure consisted in heating the porin samples at 60 °C instead of 100 °C. The Sephadex column was equilibrated with 10 mM Tris/HCl buffer, pH 7.5, containing 0.25% SDS, 0.05% NaN_3 and 100 mM NaCl. The proteins were eluted with the same buffer. Some critical steps used in this procedure have previously been reported [7,22]. The 35-kDa and 36-kDa *S. typhi* Ty 2 porins were identified by comparison with *S. typhimurium* outer-membrane proteins [7,23] in SDS/polyacrylamide gel electrophoresis (SDS-PAGE) according to Ames [24].

Chemical assays

Protein concentrations were measured by a modified method of Lowry et al. [25], and by the method of Bradford [26]. Lipopolysaccharide was determined by the thiobarbituric acid method modified by Osborn [27]. SDS was measured by Hayashi's procedure [28].

Immunizations

Rabbit antisera [anti-porin, anti-(bovine serum albumin) and anti-(*S. typhi* lipopolysaccharide)] were prepared following the immunization schedule described by Cofré et al. [29].

Erythrocyte lysis

The hemolysis experiments were carried out according to the procedure of Irmscher and Jung [15]. Briefly, 1 ml of human blood, group B, was mixed with 9 ml of 10 mM sodium phosphate buffer, pH 7.0 containing 154 mM NaCl (buffer SP). The red cells were washed twice and resuspended in 9 ml of the same buffer. From this suspension, 0.4 ml was diluted further with 5.6 ml of buffer SP and used for the hemolysis assays. 10 µl of porin suspension (final concentration around 10 nM) were added to the red-cell suspension and incubated at 37 °C, or at other temperatures as required, for 60 min. Hemolysis was measured at 546 nm. Total hemolysis was induced with 0.1 mM SDS final concentration.

In those experiments where the hematocrit was to be measured, the erythrocytes were diluted 1:1 with buffer SP. The hematocrit (percentage of red cell volume) was measured in an International microcapillary centrifuge, model MB. The hemolysis was followed using Drabkin reagent [30].

Chemical modification of proteins

Porins suspended in 200 mM Tris/HCl buffer, pH 7.5, were alkylated with 10 mM pyridoxal phosphate at 25 °C for 2 h and reduced with 0.5 mg/ml of sodium borohydride [31]. Some porin preparations in 90 mM $NaHCO_3$ buffer, pH 7.8, were also modified with 1.5% phenylglyoxal hydrate according to the procedure of Takahashi [32].

RESULTS

Purification of porins

We co-purified *Salmonella typhi* Ty 2 porins by a procedure that employed some critical steps previously reported [7,21,22]. According to migration in SDS-PAGE and solubility characteristics (Fig. 1), these proteins are homologous to the 35-kDa and 36-kDa porins found in *Salmonella typhimurium* [7,23]. It should be noted that in outer-membrane preparations of *S. typhi* Ty 2 we have not observed in SDS-PAGE a porin homologous to the 34-kDa porin of *S. typhimurium*.

The porins were obtained purified in the first peak eluted from a Sephadex G-150-120 column (Fig. 2), free of any other protein and also free of lipopolysaccharide according to the procedure of Osborn [27]. The second peak, although containing the porins, was discarded because variable amounts of lipopolysaccharide were detected. Similar results have been found by Yamada and Mizushima [33].

In order to be used in the hemolytic assays, porins were extensively dialyzed against 1 mM $NaHCO_3$ to diminish SDS

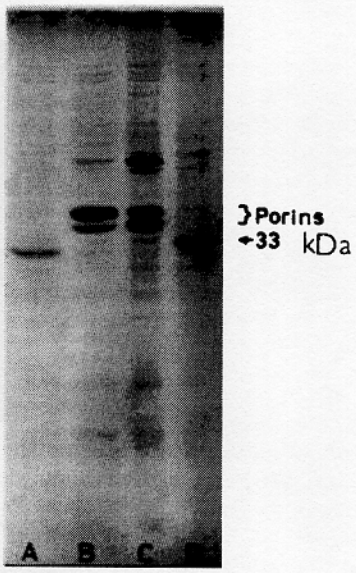

Fig. 1. *The effect of temperature on the solubilization of membrane proteins.* (A, B) Slab gel electrophoresis profiles of outer-membrane fractions from *S. typhi* Ty 2 suspended in 62.5 mM Tris/HCl buffer, pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol and 0.001% bromophenol blue [23]. (C, D) Outer-membrane fractions from *S. typhimurium* suspended as above. (A, D) Unheated sample; (B, C) heated at 100 °C for 5 min

[34]. We observed that as the SDS decreases there was an aggregation of porins resulting in the formation of insoluble oligomers. In these conditions we found an SDS concentration of 1.3 mM contaminating the porin preparation. In the hemolytic assay mixture this concentration became 2.2 µM, which is not only not hemolytic but a proven protector of red blood cells [35]. We made an effort to obtain an SDS-free porin preparation by using a drastic procedure consisting of dialysis against 6M urea and passage through a Dowex 1 X-2 column [36]. This time, the SDS concentration was 0.16 µM in the hemolytic assay, yet we still observed hemolysis. Unfortunately, this procedure yielded a poor protein recovery and was not routinely followed.

Hemolytic effect

The addition of purified porins to human erythrocytes caused their rapid lysis. This effect was observed by the use of nanomolar concentrations of porins. In calculating the molarity we have assumed that the porin oligomers formed are at least trimers. This is based on the finding made by Tokunaga et al. [9] that the smallest porin oligomers with channel-forming ability are trimers. X-ray diffraction studies, cited by the same authors, indicate that oligomers in aqueous solution when incorporated into vesicle membranes exist in the same physical state as in dodecyl sulfate.

A significant inhibition of the hemolytic capacity, around 50%, was observed if 10 µl of undiluted anti-porin serum was added to the mixture 5 min after the mixing of red cells with 30 µl of 3.2 µM porin suspension (Table 1). If the same porin suspension was pre-treated for 5 min with undiluted anti-porin serum the inhibition was complete and no hemolysis was observed. In contrast, when anti-(bovine serum albumin)serum was added to the assay mixture no inhibition of porin-induced hemolysis was observed, disproving the idea

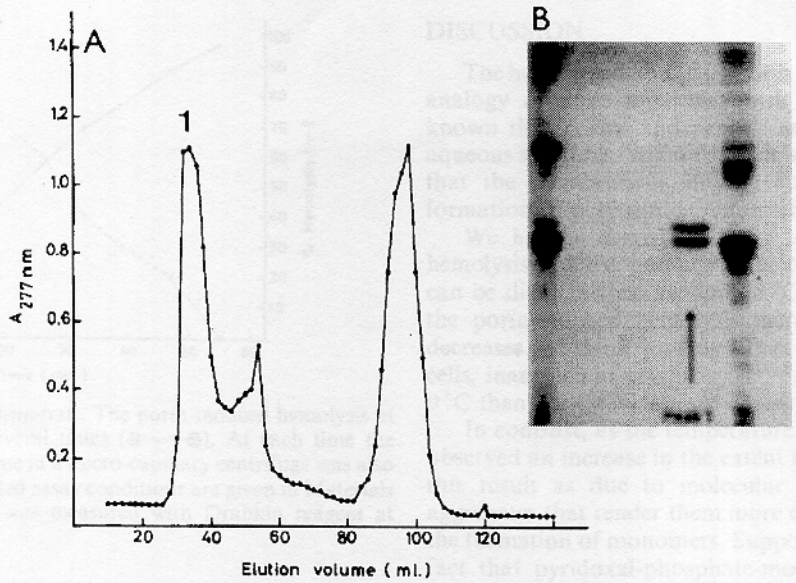

Fig. 2. *Filtration of porin fractions on Sephadex G-150-120.* (A) The experimental conditions are described under Materials and Methods. Peak 1 contained the purified 35-kDa and 36-kDa porins from *S. typhi* Ty 2, free of lipopolysaccharide. (B) Slab gel electrophoretic analysis of peak 1, compared with crude outer-membrane fractions

Table 1. *Porin-induced hemolysis inhibited by anti-porin rabbit serum* Hemolysis was measured at 546 nm after incubation at 37 °C for 60 min of a red cell suspension with the additions indicated. BSA = bovine serum albumin, LPS = lipopolysaccharide

Expt	Porin addition	Antiserum addition	Hemolysis
			%
1.	+	none	86.3 ± 4.2
2.	+	anti-porin	41.7 ± 3.2
3.	none	anti-porin	0
4.	+	anti-BSA	83 ± 4.0
5.	+	anti-LPS	71.4 ± 8.5
6.	porins pre-treated with anti-porin serum		0

that the inhibition caused by the anti-porin serum might be unspecific.

Temperature dependence of the hemolytic reaction

At a constant concentration of porins (21 nM), different extents of hemolysis were obtained depending on the temperature of the incubation mixture. As shown in Fig. 3, when the incubation was carried out at 2 °C, around 35% hemolysis was observed. Increasing the temperature of incubation resulted in a decreased hemolysis reaching the lowest hemolytic effect around 25 °C. Further increases in the incubation temperature increased the hemolysis attained, obtaining a maximum of around 70% at 37 °C. This result ruled out the possibility that the agent causing the hemolysis was contaminating SDS, since SDS used at 0.2 mM was equally effective as a hemolytic agent in the range of 2 – 37 °C [15].

Porins purified by using a heating step of 60 °C (instead of 100 °C) before Sephadex gel filtration, were also able to cause hemolysis at nanomolar concentrations and this effect was temperature-dependent (results not shown).

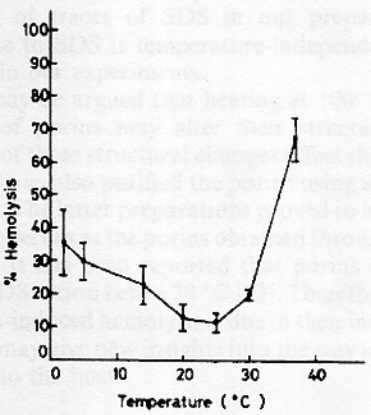

Fig. 3. *Temperature dependence of the hemolytic reaction.* Erythrocyte suspensions at temperatures ranging from 2 °C to 37 °C were added with purified porin fractions to a final concentration of approximately 21 nM and incubated for 60 min. Hemolysis was measured at 546 nm. Error bars indicate the range of values, five per data point

Hematocrit versus hemolysis

When we tried to follow the course of the hemolytic reaction by measuring the hematocrit, we found that during the first 15 min of incubation the hematocrit remained unchanged although around 35% of red cells had been lysed. At longer incubation times a sharp decrease in hematocrit values was observed (Fig. 4). We believe that this may be due to the fact that initially the red cells swell. Therefore, the disappearance of cells that are lysed is compensated by an increase in volume of the remaining cells.

Chemical modification of porins

Since it has been suggested that positive charges may be involved in the lytic activity of mellitin [37], we attempted the chemical modification of porins at lysine and arginine residues

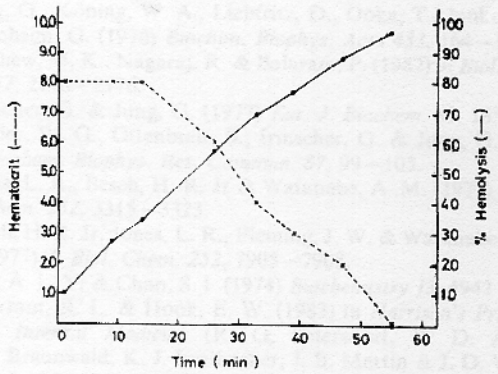

Fig. 4. *Hematocrit versus hemolysis.* The porin-induced hemolysis at 37 °C was measured at several times (●——●). At each time the percentage of red-cell volume in a micro-capillary centrifuge was also measured (× ··· ×). Detailed assay conditions are given in Materials and Methods. Hemolysis was measured with Drabkin reagent at 546 nm

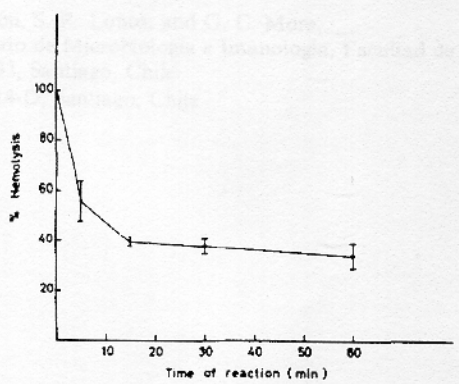

Fig. 5. *Hemolysis inhibition after chemical modifications of porins.* Porin preparations modified with pyridoxal phosphate under reductive conditions were additionally modified with 1.5% phenylglyoxal hydrate for 60 min. At intervals, samples were taken and added to erythrocyte suspensions. Hemolysis was measured at 546 nm after 30 min of incubation

using pyridoxal phosphate and phenylglyoxal respectively [31,32].

The reductive alkylation of porins with pyridoxal phosphate and sodium borohydride resulted in the disappearance of the insoluble aggregates present in aqueous solution. Dialysis against 90 mM $NaHCO_3$ to eliminate the excess of modifying reagents was also useful to eliminate SDS that had remained with the porin aggregates. We measured a lowering of SDS from 378 μg/ml to 80 μg/ml at similar protein concentrations. After the alkylation of porins, their hemolytic capacity and its temperature dependence remained unchanged (results not shown). Furthermore, the solubilized porins were now able to migrate in SDS-PAGE mainly as monomers without the heating in SDS above 70 °C, suggesting that the chemical modifications prevented stable trimer formation.

Successful inhibition of hemolysis was found when additional modifications of porins with phenylglyoxal was made. As shown in Fig. 5, after 20 min of treatment with phenylglyoxal the hemolytic effect of the porins fell to 35%. Longer treatment resulted in greater inhibition. The hemolysis was measured after 30 min of incubation of red cells with the modified proteins. We can not explain yet the hemolytic activity remaining in the modified porins.

DISCUSSION

The hemolysis induced by porin aggregates strengthens the analogy of these molecules with peptide antibiotics. It is known that porins and peptide antibiotics do aggregate in aqueous solutions because of their hydrophobic nature [9] and that the membrane-modifying activity depends upon the formation of polypeptide oligomers.

We have observed a strong temperature effect on the hemolysis which apparently is the result of two processes that can be distinguished around 25 °C. Below this temperature, the porin-induced hemolysis increases as the temperature decreases. We think this may reflect an intrinsic property of red cells, inasmuch as erythrocytes appear to be more fragile at 0 °C than 10 °C [38].

In contrast, as the temperature increased above 25 °C we observed an increase in the extent of hemolysis. We interpret this result as due to molecular rearrangements of porin aggregates that render them more effective, perhaps, towards the formation of monomers. Supporting this possibility is the fact that pyridoxal-phosphate-modified porin preparations lack the aggregate forms yet these solubilized porins were notably hemolytic. Furthermore, the alkylated porins are apparently prevented from stable trimer formation, since we found that in SDS-PAGE they migrated as monomers without the otherwise inevitable heating step above 70 °C [23].

We were able to rule out the possibility of hemolysis due to the presence of traces of SDS in our preparation, since hemolysis due to SDS is temperature-independent, which is not the case in our experiments.

Since it may be argued that heating at 100 °C during the purification of porins may alter their structure and as a consequence of these structural changes affect their hemolytic capacity, we have also purified the porins using a heating step of only 60 °C. The latter preparations proved to have the same hemolytic properties as the porins obtained through the 100 °C heating step. It has been reported that porins are generally resistant to SDS action below 70 °C [23]. Therefore, we believe that the porin-induced hemolysis is due to their intrinsic nature and this fact may give new insights into the way some bacteria cause injury to the host.

We thank A. Yudelevich for the critical reading of this manuscript and Omar Mellado for technical assistance. This research was supported by a grant from the *Dirección de Investigación, Pontificia Universidad Católica de Chile.*

REFERENCES

1. Ofek, I. & Beachey, E. H. (1978) *Infect. Immun. 22*, 247–254.
2. Peterson, P. K. & Quie, P. G. (1981) *Annu. Rev. Med. 32*, 29–43.
3. Svanborg Edén, C. & Hanson, H. A. (1978) *Infect. Immun. 21*, 229–237.
4. Swanson, J. (1973) *J. Exp. Med. 137*, 571–589.
5. Buchanan, T. M. & Pearce, W. A. (1979) in *Bacterial Outer Membranes. Biogenesis and Functions* (M. Inouye, ed.) pp. 475–514, Wiley-Interscience, New York.
6. Nakae, T. (1976) *Biochem. Biophys. Res. Commun. 71*, 877–884.
7. Nakae, T. (1976) *J. Biol. Chem. 251*, 2176–2178.
8. Nixdorff, K., Fitzer, H., Gmeiner, J. & Martin, H. H. (1977) *Eur. J. Biochem. 81*, 63–69.
9. Tokunaga, M., Tokunaga, H., Okajima, Y. & Nakae, T. (1979) *Eur. J. Biochem. 95*, 441–448.
10. Mueller, P. & Rudin, D. O. (1968) *Nature (Lond.) 217*, 713–719.
11. Boheim, G., Janko, K., Liebfritz, D., Ooka, T., Köning, W. A. & Jung, G. (1976) *Biochim. Biophys. Acta 443*, 182–189.
12. Boheim, G. & Kolb, H. A. (1978) *J. Membr. Biol. 38*, 99–150.

13. Jung, G., Köning, W. A., Liebfritz, D., Ooka, T., Janko, K. & Boheim, G. (1976) *Biochim. Biophys. Acta 433*, 164–181.
14. Mathew, M. K., Nagaraj, R. & Balaram, P. (1982) *J. Biol. Chem. 257*, 2170–2176.
15. Irmscher, G. & Jung, G. (1977) *Eur. J. Biochem. 80*, 165–174.
16. Bessler, W. G., Ottenbreit, B., Irmscher, G. & Jung, G. (1979) *Biochem. Biophys. Res. Commun. 87*, 99–105.
17. Jones, L. R., Besch, H. R. Jr & Watanabe, A. M. (1977) *J. Biol. Chem. 252*, 3315–3323.
18. Besch, H. R. Jr, Jones, L. R., Fleming, J. W. & Watanabe, A. M. (1977) *J. Biol. Chem. 252*, 7905–7908.
19. Lau, A. L. Y. & Chan, S. I. (1974) *Biochemistry 13*, 4942–4948.
20. Guerrant, R. L. & Hook, E. W. (1983) in *Harrison's Principles of Internal Medicine* (R. G. Petersdorf, R. D. Adams, E. Braunwald, K. J. Isselbacher, J. B. Martin & J. D. Wilson, eds) 10th edn, pp. 957–965, McGraw-Hill Book Company, New York.
21. Moore, D., Sowa, B. A. & Ippen-Ihler, K. (1981) *J. Bacteriol. 146*, 251–259.
22. Lugtenberg, B., Bronstein, H., Van Selm, N. & Peters, R. (1977) *Biochim. Biophys. Acta 465*, 571–578.
23. Ames, G. F. L., Spudich, E. N. & Nikaido, H. (1974) *J. Bacteriol. 117*, 406–416.
24. Ames, G. F. L. (1974) *J. Biol. Chem. 249*, 634–644.
25. Hartree, E. F. (1972) *Anal. Biochem. 48*, 422–427.
26. Bradford, M. M. (1976) *Anal. Biochem. 72*, 248–254.
27. Osborn, M. I. (1963) *Proc. Natl Acad. Sci. USA 50*, 499–506.
28. Hayashi, K. (1975) *Anal. Biochem. 67*, 503–506.
29. Cofré, G., Calderón, I. & Mora, G. C. (1978) *Infect. Immun. 20*, 161–166.
30. Drabkin, D. L. (1949) *Am. J. Med. Sci. 217*, 710–711.
31. Klein, S. M. & Sagers, R. D. (1967) *J. Biol. Chem. 242*, 301–305.
32. Takahashi, K. (1968) *J. Biol. Chem. 243*, 6171–6179.
33. Yamada, H. & Mizushima, S. (1980) *Eur. J. Biochem. 103*, 209–218.
34. Rosenbusch, J. P. (1974) *J. Biol. Chem. 249*, 8019–8029.
35. Seeman, P. (1966) *Biochem. Pharmacol. 15*, 1767–1774.
36. Weber, K. & Kuter, D. J. (1971) *J. Biol. Chem. 246*, 4504–4509.
37. Hider, R. C., Khader, F. & Talham, A. S. (1983) *Biochim. Biophys. Acta 728*, 206–214.
38. Jacobs, M. H. & Parpart, A. K. (1931) *Biol. Bull. 60*, 95–119.

I. Caldèrón, S. R. Lobos, and G. C. Mora,
Laboratorio de Microbiologia e Imunologia, Facultad de Ciencias Biológicas, Pontifica Universidad Católica de Chile,
Casilla 233, Santiago, Chile
Casilla 114-D, Santiago, Chile