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Biochimica et Biophysica Acta 1238 (1995) 72–80

BBA Biochimica et Biophysica Acta

Binding of antibodies to functional epitopes on the pore formed by *Escherichia coli* hemolysin in cells and model membranes

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Received 13 October 1994; revised 30 March 1995; accepted 26 April 1995

Abstract

Escherichia coli hemolysin (HlyA) inserts into target membranes producing a cation-selective pore. We approached the problem of determining which portions of this protein remain exposed on the side of attack by applying specific antibodies. Results obtained with resealed erythrocyte ghosts and planar phospholipid membranes were compared. The effects of one polyclonal and four monoclonal anti-hemolysin antibodies (mAbs) were studied. Using ghosts we found one mAb which strongly reduced the ion-permeability through the preinserted HlyA channels and one which clearly increased it. Experiments with planar bilayers corroborated these results by showing that the former mAb effectively promoted the closed state of the channel whereas the latter forced the HlyA channel into an open configuration. Anti-hemolysin polyclonal antibodies initially stimulated but then prevented channel opening, indicating they contained clones able to act on both these channel determinants. They were effective only when applied on the same side as the hemolysin indicating that the epitopes were exposed to that side. Finally, the antigenic epitopes of three of the mAbs were localised on the HlyA molecule by using different mutants (amber and frame shift mutants and hemolysin gene hybrids).

Keywords: Hemolysis; Ion channel; Phospholipid bilayer; Monoclonal antibody; Erythrocyte ghost; Epitope mapping

1. Introduction

Escherichia coli hemolysin (HlyA) is the prototype of a family of pore-forming cytotoxins elaborated by Gram-negative organisms including *E. coli, Proteus, Morganella, Pasteurella, Bordetella* and *Actinobacillus* species [1,2]. These toxins are secreted by a common, not yet fully understood, mechanism [3]. To become activated, they require a post-translational modification consisting of an acylation step [4,5]. A prominent feature of these toxins is the presence of regions containing homologous glycine-rich repeated nonapeptides, which have been found also in other non-toxic bacterial proteins, e.g., *Pseudomonas, Serratia* and *Erwinia* proteinases [6–8] and *Rhizobium* nodulating protein NodO [9]. These repeats form short β-strands organised in an unusual spring-like structure, called parallel β-barrel or β-superhelix, which binds Ca^{2+}-ions in a stoichiometry of one ion per repeat [6]. It was suggested that Ca^{2+} has a structural role in stabilising this peculiar folding [6], and Ca^{2+}-ions were found to be necessary both for cell-binding and for expression of lytic activity by HlyA [10,11].

HlyA has a wide spectrum of cytocidal activity attacking granulocytes [12,13], monocytes [14], endothelial cells [15], and renal epithelial cells [16]. The leukocidal action of other members of this toxin family has also been documented (reviewed in [1,2]). Basic functional aspects of the family have been most thoroughly investigated with

Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; RBC, red blood cells; TLC, thin-layer chromatography; PEG, poly(ethylene glycol); SPQ, 6-methoxy-*N*-(3-sulfopropyl)quinolinium; DIDS, diisothiocyanostilbene-2,2′-disulfonic acid; HlyA, *Escherichia coli* hemolysin; mAb, monoclonal antibody; PLM, planar lipid membrane; ELISA, enzyme-linked immunoadsorption assay.

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SSDI 0005-2736(95)00113-1

HlyA [17,18] and it was found that erythrocytes, and even planar lipid membranes (PLM), are valuable models for studying toxin action [19–22]. HlyA transmembrane pores have been sized to 1–2 nm functional diameter both in RBC and in PLM [19,23]. They display cation selectivity, voltage-and pH-dependence, and are sensitive to proteinases [19,24,25].

Once bound to a membrane HlyA behaves as an intrinsic protein as it cannot be extracted from RBC membranes without the use of a detergent [23] and the pore formed in a PLM, either in the open or in the closed configuration, cannot be detached from the lipid film by thorough perfusion with toxin-free solution [26]. In a PLM the channel is disposed asymmetrically in the membrane because proteolytic enzymes digest it only when applied to the *cis* side [19,24]. Similar results were obtained also in the case of HlyA-pores on RBC membranes [25], implying that the membrane bound configuration of HlyA might be similar in both natural and model membranes.

In this work we have used different anti-HlyA antibodies to determine which portions of the toxin remain exposed to the *cis* side of the membrane after channel insertion into RBC and PLM. Stimulatory, inhibitory and indifferent monoclonal antibodies have been identified and the localisation of their epitopes was investigated.

2. Materials and methods

2.1. Chemicals

HlyA was prepared as described [23], lyophilised and stored at −20°C. These preparations were stable for several months. Anti-HlyA polyclonal antibodies (IgG) were raised in rabbits and purified by affinity chromatography. Anti-HlyA mAbs were prepared as described [27]. The fluorescent marker 6-methoxy-*N*-(3-sulfopropyl)quinolinium (SPQ) was purchased from Calbiochem. Saturated egg phosphatidylcholine (PC) came from P.L. Biochemicals, brain phosphatidylserine (PS) was from Calbiochem. Both lipids were more than 99% pure by TLC.

The following buffer solutions were used. Buffer PBS: NaCl 150 mM, sodium phosphate 5 mM, pH 8.0 (isotonic solution). Buffer 5P8: sodium phosphate 5 mM, pH 8.0 (hypotonic solution). Buffer A: sucrose 100 mM, KCl 100 mM, Hepes-Tris 50 mM, pH 7.0 (internal chloride solution). Buffer B: sucrose 100 mM, K_2SO_4 66 mM, Hepes-Tris 50 mM, pH 7.0 (external sulfate solution). Buffer C: KCl 100 mM, Hepes-Tris 5 mM, pH 7.0.

2.2. Assays

Determination of hemolytic activity

After reconstitution in water, the hemolytic activity of HlyA was tested on human erythrocytes as described [23]. Briefly: samples of HlyA were serially diluted into a 96 wells microtiter plate (each well contained 50 μl Buffer PBS and the toxin was twofold-diluted at each step). An equal volume of human RBC (2.5% (v/v) in Buffer PBS) was added to the wells and titers were read visually after one hour incubation at 37°C and expressed as the reciprocal of the last dilution giving ≥ 50% hemolysis. Hemolytic activity was usually around 1000–2000 hemolytic units/ml (HU/ml). Under these conditions we found that the relation between hemolytic activity and molar concentration of HlyA, as it was calculated from its UV absorption using an extinction coefficient ϵ_{280} of 73 960 M^{-1} cm^{-1} (evaluated from the primary sequence) was 1 HU/ml = 10 nM ≈ 1 μg/ml.

Preparation of SPQ loaded erythrocyte ghosts and assay of chloride / sulfate exchange

Pink erythrocyte ghosts were resealed in the presence of 4 mM SPQ by a procedure adapted from [28] that we extensively described earlier [25]. Thereafter, they were washed twice in cold Buffer A (10 min centrifugation at 22 000 × *g*), in order to remove untrapped SPQ. The final pellet had a count of about $3 \cdot 10^9$ ghost/ml and a protein content of 3.3 mg/ml as determined by the method of Lowry [29]. It was resuspended in 3 volumes of Buffer A and kept on ice.

Cl^-/SO_4^{2-} exchange was measured using a Jasco FP550 spectrofluorimeter essentially as described [25]; excitation was set at 320 nm and emission at 445 nm. Briefly: 5 μl of SPQ-loaded ghosts were added to a quartz cuvette containing 1 ml of the sulfate buffer (Buffer B) at 25°C. The release of chloride from the interior of the ghosts (in exchange with sulfate) resulted in an increase of SPQ fluorescence since the chloride-induced quenching was removed [30]. Maximal SPQ fluorescence was determined each time by addition of 1 mM Triton X-100 and used as 100%.

Binding of monoclonal antibodies to HlyA-permeabilized RBC

Toxin-treated erythrocyte membranes were prepared mixing rabbit erythrocytes (10^9 cells per ml in PBS) with HlyA (final concentration 1000 HU/ml) and incubating for 1 h at 37°C. Pelleted cells and membranes were washed twice in PBS (16 000 × *g*, 10 min), and thrice in Buffer 5P8 (which converted even unlysed cells to ghosts). In controls, toxin was omitted and cells were lysed simply by exposure to the hypotonic buffer. The final pellet was resuspended in PBS (original volume) and 500 μl aliquots were transferred to Eppendorf tubes for ELISA measurements. Prior to use, the walls of these tubes were blocked with 0.5% gelatin in PBS. Purified mAbs in PBS were added to each tube (final concentration: 10 μg/ml) and incubated for 60 min at 37°C. The membranes were washed thrice in PBS plus 0.5% gelatin. Binding of mAb was detected incubating with a second antibody (rabbit anti-

mouse-IgG alkaline phosphatase conjugated, Dakopatts, Hamburg, diluted 1:1000 in PBS) in a total volume of 500 μl/tube for 45 min at 37°C. Thereafter, a substrate (*p*-nitrophenylphosphate; Sigma, Munich, Germany) was added for approx. 5 min and the enzyme reaction was stopped by spinning the membranes down; the colour development in the supernatant was read photometrically at 405 nm (SLT Labinstruments, Overath, Germany).

Antibody effects on HlyA-permeabilized ghosts

Ghosts were resealed in the presence of 4 mM SPQ as described above (1 h incubation at 37°C). Before removing the untrapped fluorophore, they were further incubated 15 min with HlyA at 37°C. Unbound toxin was removed by washing three times (10 min, $14000 \times g$) in Buffer A containing 4 mM SPQ. After this step a sample of the HlyA-treated ghosts was used as control, the rest was subjected to incubation with the antibodies for 30 min (room temperature). Unbound antibodies and untrapped SPQ were finally removed by washing twice and resuspending in Buffer A. Compared to the starting sample of unsealed ghosts, the final pellet was tenfold concentrated. During this procedure all the buffer solutions were supplemented with 3 mM PEG (Fluka, M_r 6000) to prevent ghost swelling [25]. The amount of SPQ retained was estimated by mixing 5 μl of ghosts with 1 ml of Buffer B in a cuvette and following the time-course of the fluorescence increase in a fluorimeter as described above. Further controls were prepared either omitting the toxin or adding first polyclonal antibodies and then HlyA.

Experiments with planar lipid membranes (PLM)

PLM were prepared by apposition of two lipid monolayers on a hole (0.2 mm in diameter) punched in a 12 μm thick Teflon septum separating two 4 ml chambers containing Buffer C, essentially as described [24]. The hole was pretreated with n-hexadecane and the monolayers were spread from a 10 mg/ml lipid mixture (PC/PS in a 1:1 molar ratio) dissolved in n-hexane. HlyA was added only on one side of a preformed stable bilayer (called the *cis* compartment). Membrane current, under voltage clamp conditions, was converted to voltage by a virtual grounded operational amplifier (Burr Brown OPA 104C). The *cis* compartment was connected to the virtual ground and voltage signs referred to it. Current is defined positive when cations flow into this compartment. The baseline conductance of these membranes was never larger than 50 pS. Ag-AgCl electrodes, directly immersed into the electrolyte solution, were used. Experiments were performed at room temperature.

Localisation of anti-HlyA mAb epitopes

The localisation of the anti-HlyA epitopes recognised by the four mAbs was determined as previously described, i.e., by immunoblotting using the antibodies as probes against various HlyA recombinant strains [31]. In addition to the recombinant strains used in that earlier study, several new deletion mutants of HlyA [32] and hybrid genes composed of *hly*A and the *Pasteurella haemolytica* leukotoxin (*lkt*A) [33] were employed. None of the anti-HlyA mAbs used here cross reacted to LktA. Therefore, we

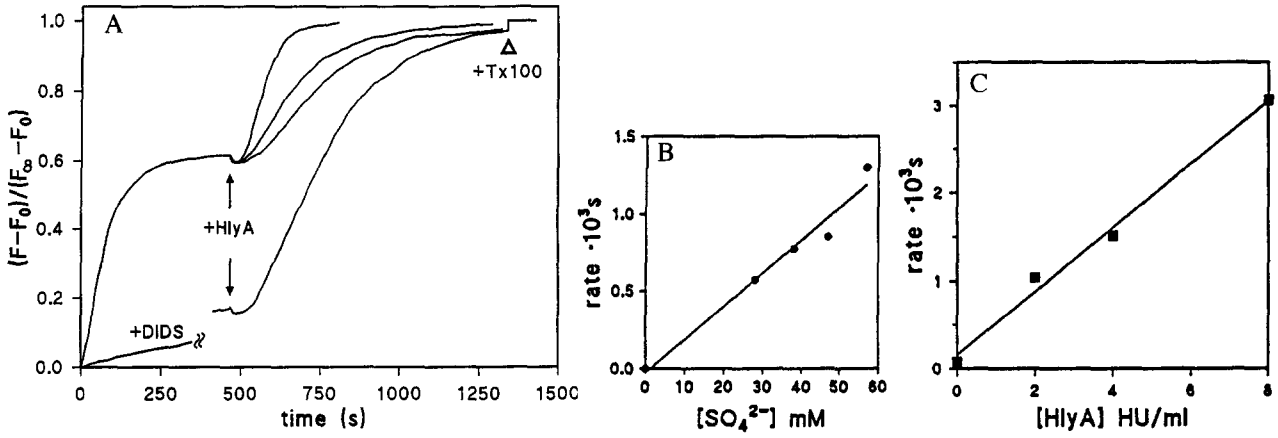


Fig. 1. Anion exchange through human resealed erythrocyte ghosts before and after exposure to *E. coli* hemolysin. At time zero 5 μl of SPQ-loaded resealed ghosts, washed in chloride buffer (Buffer A) were added to a cuvette containing 1 ml of sulfate buffer (Buffer B). The final protein concentration was 5.5 μg/ml. Chloride release from the interior of the ghosts (in exchange with sulfate) resulted in an increase of the fluorescence as the chloride-induced quenching of the internal fluorophore was relieved [30]. Reported is the time course of the fluorescence increase $F - F_0$ (where F and F_0 are the fluorescence at time t and time zero, respectively) normalized by dividing by its maximal value, obtained with 1 mM Triton X-100 ($F_\infty - F_0$). Lower trace was in the presence of 2 μM DIDS which almost abrogated the exchange. (The dependence of the initial rate of exchange on the external concentration of sulfate is shown in (B).) At the position indicated by the arrows, different doses of HlyA were added to the cuvette (2, 4 and 8 HU/ml, from bottom to top, in the upper three traces). The sustained fluorescence increase was due to the formation of pores into the resealed membranes and was unaffected by DIDS (lower trace, 8 HU/ml). (The initial rate of HlyA-induced release is shown in (C) to depend linearly on the toxin concentration.) The final addition of 1 mM Triton X-100, to obtain 100% SPQ dequenching, is indicated by an open arrow-head.

assumed that the reactivity of a test mAb to a LktA-HlyA hybrid protein localised the mAb epitope to the portion of HlyA present in that hybrid.

3. Results and discussion

3.1. HlyA-induced opening of pores in resealed ghosts

When resealed erythrocyte ghosts, loaded with SPQ and its quencher chloride, were transferred to a sulfate solution the fluorescence of the internally entrapped SPQ gradually increased with time (Fig. 1A). This was due to the exchange of internal chloride with external sulfate promoted by the band 3 protein [25]. In fact, the release was blocked by DIDS, a well known inhibitor of the RBC anion exchanger [34], and its rate was proportional to the external concentration of sulfate (Fig. 1B).

If HlyA was added to the resealed ghosts, after their transfer to the sulfate solution, a larger and faster increase of the fluorescence appeared immediately and proceeded to a steady state at which Triton X-100 did not produce much more fluorescence increase (Fig. 1A). This effect was independent of the band 3 protein (it was not affected by DIDS) and its rate was proportional to the concentration of HlyA added (Fig. 1C). Hence, it could be attributed to the formation of toxin-induced ion channels into the ghost membrane, in analogy to the case of RBC and model membranes [19,23,35].

Polyclonal anti-HlyA antibodies were able to counteract this effect in a dose dependent manner (Fig. 2). Two mechanisms may concur to produce the inhibitory effect shown: the antibody may prevent toxin binding, or they may prevent transport through the toxin pores. In the following we will try to discriminate between these two possibilities.

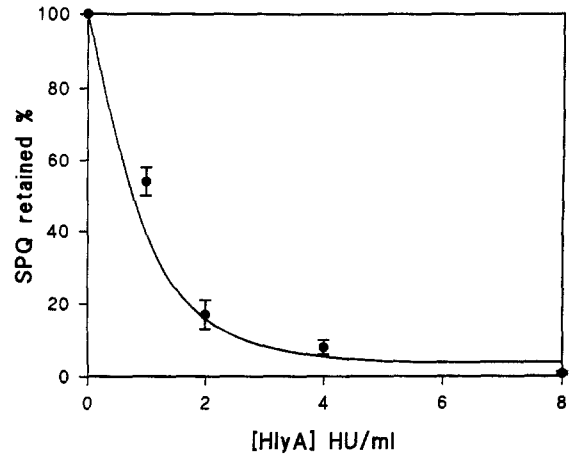


Fig. 3. Capacity of resealed erythrocytes exposed to HlyA to retain SPQ. Ghosts resealed in the presence of 4 mM SPQ were incubated 15 min with different concentrations of HlyA at 37°C and then washed thrice in Buffer A. The amount of SPQ retained was determined by mixing 5 μl of ghosts with 1 ml of Buffer B and following the time-course of the fluorescence increase as in Fig. 1. 100% corresponds to controls where the toxin was omitted. Solid line was drawn by eye.

3.2. Effects of anti-HlyA antibodies on preformed pores

Resealed ghosts

HlyA pores were large enough to permit efflux of SPQ out of the ghosts. In fact, if erythrocyte ghosts resealed in the presence of SPQ were incubated with increasing concentrations of HlyA, before removing the external fluorophore, they became progressively unable to retain SPQ (Fig. 3). We found that at 8 HU/ml each ghost carried enough HlyA-induced pores to be completely leaky to the fluorescent molecule. This is not surprising, since SPQ is a cation and has a molecular weight of 300 Da which is well below the predicted cut-off of the HlyA channel (around 2000 Da [23]).

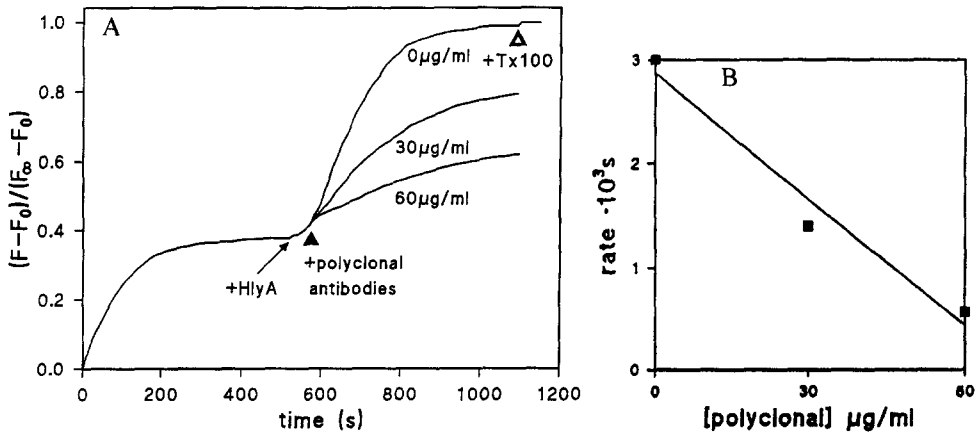
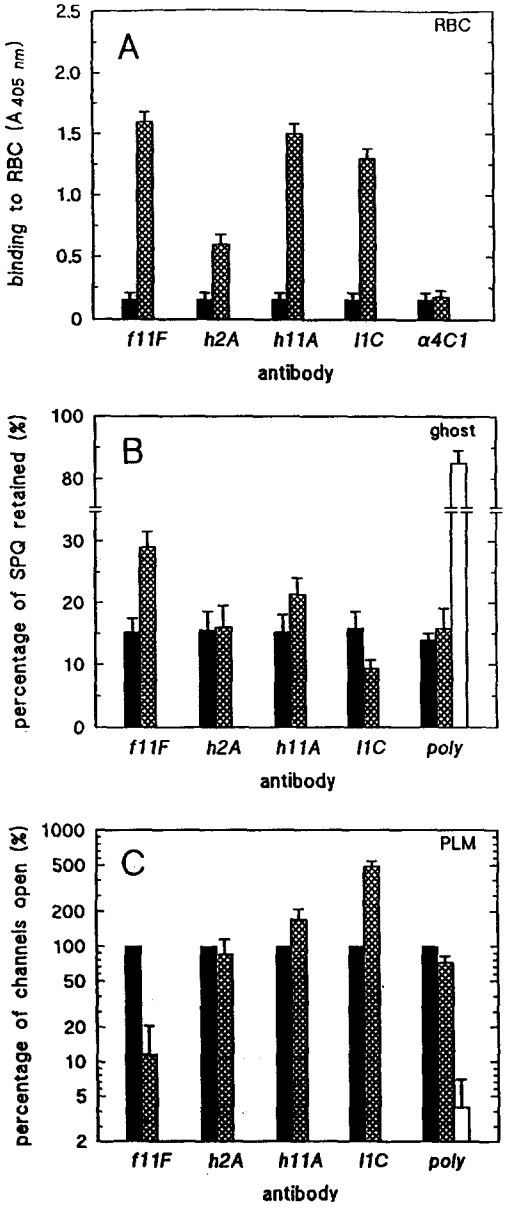


Fig. 2. Effects of polyclonal anti-HlyA antibodies on the rate of ghost permeabilisation by HlyA. (A) Same as Fig. 1 except that soon after addition of HlyA (8 HU/ml), different doses of anti-HlyA polyclonal antibodies were added to the cuvette (where indicated by the arrow-head). (The initial rate of HlyA-induced release is shown in (B) to be inversely related to the antibody concentration.) Also in this case, 100% SPQ dequenching was obtained adding 1 mM Triton X-100 (indicated by an open arrow-head). Other experimental conditions as in Fig. 1.

Interestingly, this procedure appeared to be suited to estimate the number of open channels present in the ghosts. Using this approach we then investigated the effects of monoclonal and polyclonal anti-HlyA antibodies on preformed pores (Fig. 4). After incubation with the toxin the ghosts were washed (in the presence of SPQ) and further incubated with the antibodies. Finally, external SPQ was removed and the amount of SPQ retained was determined.

Polyclonal antibodies did not reduce significantly the membrane permeability mediated by preformed HlyA pores (Fig. 4B), although they were able to protect the ghosts almost completely if added before the incubation with the toxin (which is consistent with the results in Fig. 2), and to prevent RBC hemolysis (not shown).



Four anti-HlyA mAbs were then tested. Although none of these mAb could protect RBC from hemolysis, they all bound to washed membranes of HlyA-lysed RBC (Fig. 4A). When tested for their effect on preformed pores, they behaved differently (Fig. 4B). The clearest effect was obtained with mAbs *f11F* and *l1C*. The former inhibited permeability whereas the latter promoted it. Of the other two, *h2A* had almost no effect, whereas *h11A* had a protective effect similar, albeit less pronounced, to that of *f11F*.

Planar lipid membranes

These effects could be understood in more detail by using a simplified membrane model such as the planar lipid membrane. In these membranes HlyA forms ionic channels which are open most of the time at negative voltages but may be closed by application of a positive voltage [19,24]. None of the four mAbs could prevent the formation of pores in the bilayer even if preincubated with HlyA. Nonetheless when applied to preformed HlyA pores they produced distinct effects. The clearest effect was again obtained with mAbs *f11F* and *l1C*. MAb *f11F* led to closing of the preformed channels either at negative or at positive applied voltages (Fig. 5A), whereas mAb *l1C* promoted further opening of HlyA pores at negative voltages (Fig. 5B). Both of the other two mAbs, *h2A* and *h11A*, had very little effect (Fig. 4C).

These results, consistent with those obtained using resealed ghosts, indicated that the protective effect of mAb *f11F* was due to the fact that its binding to the HlyA pores either closes the pores by steric hindrances or increases their probability to be in the closed state independently of the applied voltage. Similarly the permeability-stimulating

Fig. 4. Interaction of four different anti-HlyA monoclonal antibodies (*f11F, h2A, h11A, l1C*) with membrane bound HlyA. (A) ELISA quantitation of the binding of the mAbs to HlyA-hemolysed RBC membranes (shaded bars). Full bars represent a control experiment in which hypotonically-lysed RBC membranes were incubated with all four the anti-body clones at the same time. α4C1 was a second control experiment in which HlyA-lysed RBC membranes were incubated with a mAb directed against α-toxin of *S. aureus*. The ELISA was developed with a second antibody against mouse IgG (alkaline phosphatase conjugated) and absorbance was read at 405 nm. (B) Relative amount of SPQ retained in the internal compartment of ghosts exposed (before removing the untrapped fluorophore) to 2 HU/ml of hemolysin for 15 min at 37°C, full bars, or to the same amount of toxin followed by 0.3 mg/ml monoclonal or 0.06 mg/ml polyclonal antibodies (after removing the unbound toxin) for 30 min at room temperature, shaded bars. The amount of SPQ retained was calculated as in Fig. 3. 100% was given by control experiments with no toxin and no antibody added. Empty bar was for ghost incubated with polyclonal anti-HlyA antibodies before adding the toxin. (C) Relative amount of HlyA-channels which were found open at steady state in a planar lipid membrane clamped at −20 mV after exposure to 10 HU/ml of hemolysin (full bars = 100%) or to the same amount of toxin followed by 0.025 mg/ml monoclonal or 0.01 mg/ml polyclonal antibodies for 30 min (shaded bars). Empty bar was the effect of preincubating HlyA with polyclonal antibodies. Other experimental conditions as in Fig. 5.

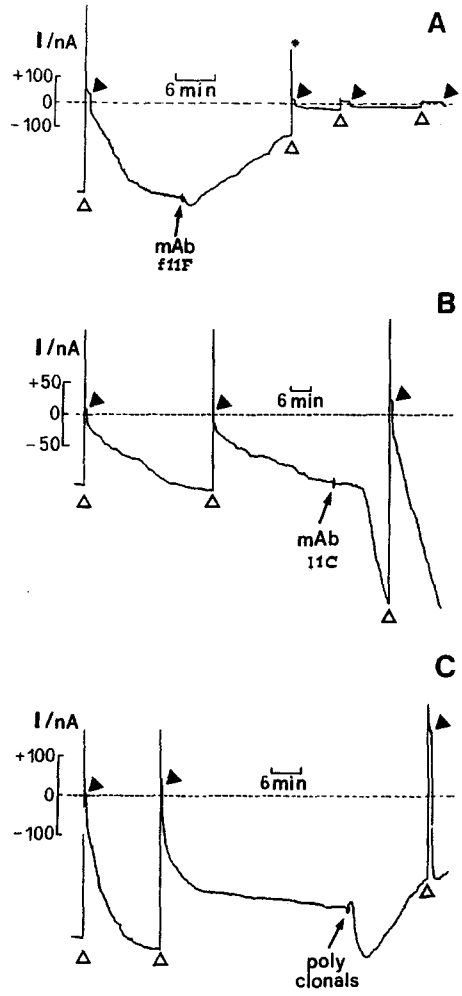


Fig. 5. Effects of different anti-HlyA antibodies on HlyA-pores present in a planar lipid membrane. A stable PC/PS PLM was incubated with 10 HU/ml HlyA (added only to the *cis* compartment). Ionic channels were soon formed into the lipid film whose opening and closing properties were determined by the applied voltage. Open arrow heads indicate switching the clamp voltage from −20 mV to +40 mV, closed arrow heads indicate switching from +40 mV to −20 mV. After the transition to the negative voltage the pores slowly open to reach a steady state. Immediately after the transition to the positive voltage the pores are open, but then they close very quickly and almost completely, as indicated by the vertical decay of the current. Where indicated 0.025 mg/ml of different antibodies were added to the *cis* compartment which was continuously stirred. (A) MAb *f11F* induced the closure or block of preformed HlyA channels even at a negative applied voltage which usually keeps the channels open. However, *f11F*-induced blockage was faster when the channels were previously brought into the closed state by a positive voltage: note that after the very short transition to +40 mV (denoted with an asterisk) quite a few channels were able to reopen at the negative voltage. (B) MAb *1C* promoted further opening of HlyA pores at negative voltages but did not prevent their voltage-induced closing at +40 mV. (C) Polyclonal antibodies (despite being fully protective if preincubated with the toxin) induced a complex effect which may be regarded as the sum of the two effects shown in (A) and (B). They first stimulated new opening of pores but then induced closing of the channels. They failed to produce any effect when applied on the *trans* side (not shown). In each panel the dashed line indicates zero current. Experiments were performed at room temperature.

effect of mAb *11C* could be attributed to its ability to promote the permanence of HlyA channels in the open state.

Polyclonal antibodies were strongly protective if preincubated with the toxin (Fig. 4C), but induced a complex effect when applied to preformed pores (Fig. 5). This effect may be regarded as the sum of those observed with mAb *f11F* and *11C*. They first stimulated new opening of pores, but then induced a closing of the channels (Fig. 5C). This result suggested that the polyclonal antibody may contain clones able to bind to both the structural determinants responsible for the closing and opening effect of mAbs *f11F* and *11C* respectively. When applied to the *trans* side of the membrane (i.e., opposite to the toxin) the polyclonal antibody did not produce any effect, indicating that the epitopes are only exposed to the *cis* side.

3.3. Mapping the anti-HlyA mAb epitopes

We then tried to localise the epitopes of the four mAbs on HlyA using the procedure described in [31], i.e., immunoblotting against different HlyA mutants, as listed in Table 1. We found that these epitopes were all independent of the HlyC modification of HlyA, since both activated and non-activated HlyA reacted in all cases (not shown). Their precise localisation however, was not without problems (Fig. 6). Ideally, an epitope is localised by the retention of the reactivity of the corresponding mAb to smaller and smaller peptide fragments. Yet, with these four mAbs, we often observed that reactivity was lost in some

Table 1
HlyA derivates used for localisation of mAb epitopes by immunoblotting

Strain	Number for Fig. 6	Portion of HlyA present	Notes	Ref.
WAM589	–	1–1023	(a)	
WAM783	–	1–1023	(b)	[31]
WAM619	1	1–829	(c)	[31]
WAM676	2	1–726	(c)	[31]
WAM841	3	161–1023	(d)	[31]
WAM802	4	626–1023	(d)	[31]
WAM713	5	1–726, 829–1023	(e)	[31,32]
WAM1233	6	1–672, 746–1023	(e)	[31,32]
WAM1263	7	1–625, 674–1023	(e)	[31,32]
WAM1127	8	392–1023	(f)	[33]
WAM1161	9	1–391	(g)	[33]
WAM1043	10	564–1023	(f)	[33]
WAM1046	11	1–563	(g)	[33]
WAM1025	12	740–1023	(f)	[33]
WAM1022	13	1–739	(g)	[33]

[a] HlyA wild type.
[b] HlyA synthesised in the absence of HlyC activation.
[c] HlyA frameshift mutants.
[d] Hlya secreted C-terminal fragments.
[e] HlyA inframe deletion mutants.
[f] LktA/HlyA hybrids.
[g] HlyA/LktA hybrids.

mutant forms that independent evidence indicated should retain the epitope.

The epitope for *f11F* must reside in the carboxy-terminal 40 kDa HlyA fragments because it reacts to the WAM802 secreted form of HlyA. In addition, because this mAb reacts to the $LktA_{1-732}$-$HlyA_{740-1023}$ hybrid, the epitope appears to reside in the HlyA glycine-rich repeat region or further toward the C-terminus. The problem is how to interpret the lack of reactivity of this mAb to either the HlyA-BgIII frameshift ($HlyA_{1-829}$) or the inframe HlyA deletion lacking residues 726–829. The *f11F* epitope may actually reside in $HlyA_{829-1023}$ region and the inframe deletion lacking most of the repeats folds unusually to obscure its epitope. Alternatively, the epitope may have a structure made of discontinuous elements involving the repeats and C-terminal portion of the molecule, or it may very well be a continuous epitope around the $HlyA_{829}$ position with portions of it missing both in the frameshift and inframe mutants. Lastly, although the size of the HlyA fragments reported in Table 1 was consistent with their molecular weight as determined by SDS-PAGE, because of the limited precision of this method we cannot exclude

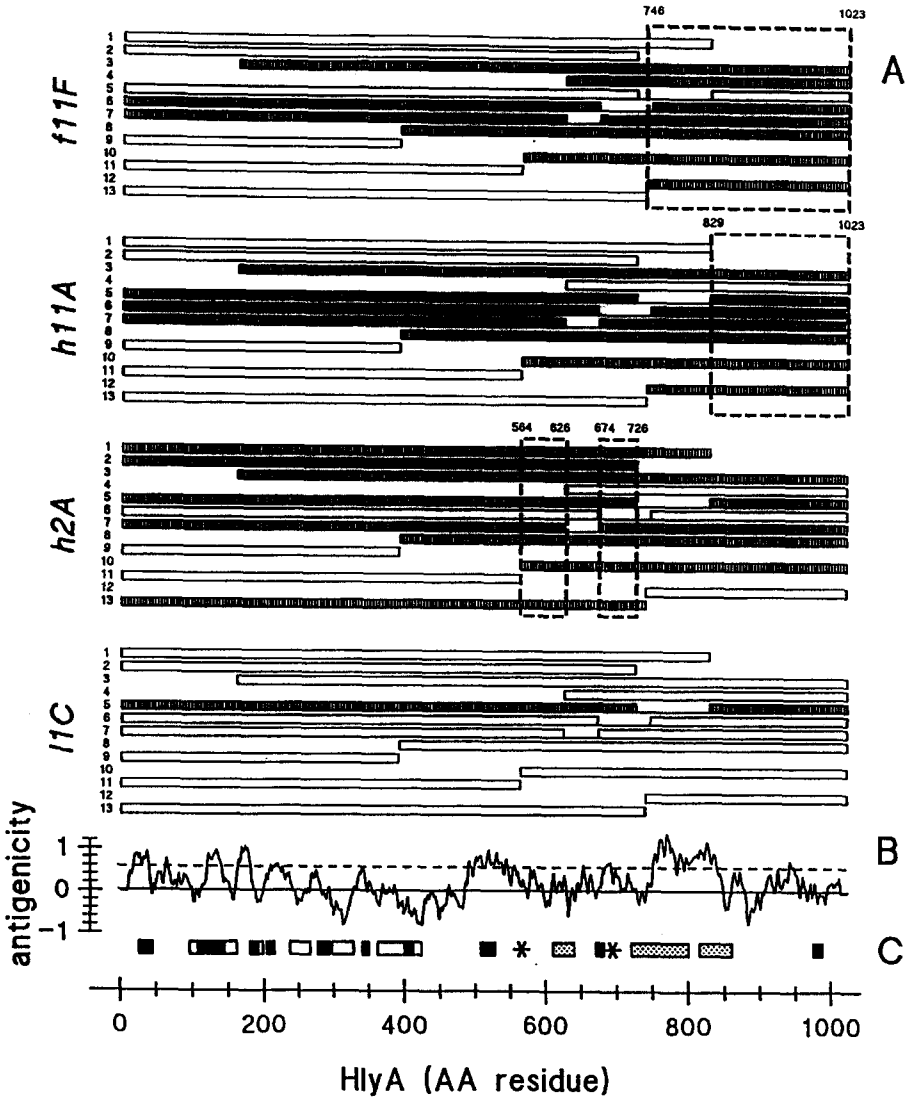


Fig. 6. Mapping of the four mAbs' epitopes. (A) Reactivity of the mabs to different HlyA mutants (numbered 1 to 13 as listed in Table 1), was studied by immunoblotting. Dashed bars represent constructs which positively reacted to a given mAb, empty bars those that were negative. Note that only the HlyA part of the molecule was reported in the case of HlyA-LktA hybrids. Ideally each mAb is mapped to a region which intercepts all positive bars and no negative one, however this was never possible. Dashed boxes indicate the most probable epitope location, i.e., a region containing all positive and at most one negative construct. All mAbs reacted to WAM 589 and WAM 783, i.e., acylated and non-acylated mature HlyA. (B) Average antigenicity calculated using the chain-flexibility scale introduced by Karplus and Schulz [36], (with a 21 AA window). The scale is based on the individual temperature factors of the C_2 atoms of AA belonging to a database of 31 proteins of known three dimensional structure. Most antigenic stretches are those exceeding the dashed line, which represents the sum of the average flexibility plus its S.D. (C) Location of some structural features along the HlyA molecule: open and closed bars, represent hydrophobic and amphipathic α-helices, respectively [20]; shaded bars, three groups of glycine-rich repeats [44]; asterisks, positions at which the mature molecule is acylated [5].

the possibility of undetected protease processing of short segments at their termini. Taking all this into account, the most conservative interpretation of the *f11F* blotting data would put its epitope within the $HlyA_{740-1023}$ region.

In the case of *h11A*, the reactivity to the WAM1025 $LktA_{1-732}$-$HlyA_{732-1023}$ hybrid indicates its epitope lies between $HlyA_{740-1023}$. The retention of reactivity to the HlyA deletion lacking position 727 through 828 indicates that the epitope lies in the $HlyA_{829-1023}$ region. The irregularity to the reactivity of *h11A* is its failure to bind in immunoblots to the 40 kDa carboxy-terminal fragment secreted by WAM802.

The epitope for *h2A* lies either in the $HlyA_{564-625}$ or $HlyA_{673-726}$ regions. It reacts to the WAM676 frameshift mutant covering residues 1–726 and to the WAM1043 $LktA_{1-554}$-$HlyA_{564-1023}$ hybrid. It also reacts to the WAM1263 deletion which lacks amino acids 626 through 673. Therefore, its epitope is most likely to reside on either side of the deletion but not between position 1 through 563 or 726 through to 1023.

The most problematic was the *11C* mAb which reacted to just three of the different HlyA forms: wild-type HlyA (activated or not) and one deletion mutant lacking eleven of the thirteen glycine-rich repeats. Because this mAb does readily react to native HlyA by ELISA (Fig. 4A), these results suggest that the *11C* epitope is likely to involve a discontinuous epitope that is not dependent on the glycine rich repeats or the HlyC acylation of HlyA, but which is sensitive to deletions or substitutions throughout the HlyA molecule.

The results of mapping were compared to a theoretical profile of the average antigenicity along the hemolysin sequence obtained by the method of Karpplus and Schulz [36] (Fig. 6B). It appears that the two inhibitory mAbs (*f11F* and *h11A*) may in fact both bind to the repeat region which is the most antigenic. This region (Fig. 6C) is not believed to be itself part of the HlyA pore, which is instead most probably formed by a number of hydrophobic and amphiphilic α-helices which are grouped in the region from residue 100 through residue 412 [20] (a region highly conserved among the parent leukotoxins and known to be essential for the lytic, albeit not for the binding step [37,38]). Apparently, deletion of the repeats leaves unaffected the pore-forming ability of HlyA [11], however, it is still possible that binding of a mAb to this region causes a conformational change of the molecule such that the pore becomes blocked [39–41]. This is in line with the finding that a mutated hemolysin, with an insertion into the repeat region, is unable to form stable pores (G. Menestrina and R. Welch, unpublished results). It also provides an explanation for the blocking effect of Zn^{2+} [42] which can replace Ca^{2+} in at least one binding site on the repeat region [6,43] and thus elicit a conformational change of the molecule. In any case our results would indicate that this region remains located *cis*, i.e., on the same side from which the toxin has approached the membrane.

Acknowledgements

This work was financially supported by the Italian Ministero dell'Università e della Ricerca, by the Consiglio Nazionale delle Ricerche (CNR), and by U.S. Public Health Services Grant AI 20323. G.M. and R.A.W. acknowledge also a NATO Collaborative Research Grant No. 920136. M.R. and M.D.S. were the recipients of fellowships from the CNR (No. 201.02.38 and 201.02.45-21.02.05, respectively). C.P. had a fellowship from the Fondazione Trentina per la Ricerca sui Tumori.

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