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Immunogenicity of Ribosomes from Enzymatically Lysed *Streptococcus pyogenes*

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Ribosomal fractions isolated from *Streptococcus pyogenes* by physical and enzymatic disruption of the cell wall were found to provide protection in mice against challenge with the homologous M type. Although ribosomal fractions isolated by physical disruption of the cells also provided protection against challenge with several heterologous M types, ribosomal fractions from enzymatically lysed cells did not provide protection against any of the heterologous M types. Ribosomes isolated by either method were found to be contaminated with cell surface proteins. Chemical analysis of the ribosomes showed a greater protein: ribonucleic acid ratio in ribosomes from physically disrupted cells than in ribosomes from enzymatically disrupted cells (2:1 versus 1:1). Antisera to ribosomes isolated from physically disrupted cells detected many more antigenic determinants on ribosomes isolated from enzymatically disrupted cells than did the corresponding homologous antisera. Immunodiffusion analysis suggested that ribosomes isolated from physically disrupted cells may contain cell wall antigenic determinants which are present on ribosomes isolated from enzymatically disrupted cells in a partially degraded form. Washing of ribosomes in high-molarity salt solutions suggested that some of the contaminating cell wall proteins are tightly bound to the ribosomes.

Ribosomal fractions have been recognized for their protective effects since Youmans and Youmans first demonstrated the protective ability of ribosomal fractions isolated from *Mycobacterium tuberculosis* (36-38). Although ribosomes from a number of organisms have been shown to confer protection to challenge with the homologous organisms (2, 7, 11, 19, 22, 28, 31, 32, 35), the chemical nature of the protective immunogen appears to vary from organism to organism (19, 24, 25, 29, 31-33). Even though ribosomal vaccines from several systems have been shown to be contaminated with cell wall material (4, 10, 11, 18), the cellular location of the protective immunogen in ribosomal vaccines remains unknown.

Ribosomal vaccines from *Streptococcus pneumoniae* (30, 32) and *Streptococcus pyogenes* (28) have been shown to provide protection against challenge with the homologous organism. However, ribosomal vaccines from these organisms are unique in that they also confer protection against challenge with heterologous serotypes of pneumococci and streptococci (28, 30). Although protein has been shown to be the effective immunogen in both of these ribosomal vaccines (28, 30), little is known about the localization of the protective immunogens. Since the protective ribosomal preparations of many organisms are contaminated with cell wall material and the exact location of the protein antigen providing protection is unknown, an investigation was conducted to determine whether enzymatic disruption of streptococcal cells had any effect on the protection induced by ribosomes isolated from *S. pyogenes*.

MATERIALS AND METHODS

Animals. Swiss-Webster mice (6 to 8 weeks old) weighing from 18 to 20 g were obtained from a mouse colony maintained by the Department of Microbiology, University of Iowa, Iowa City.

Cultures. Group A, type 14S23 *S. pyogenes* was obtained from Rebecca Lancefield (Rockefeller University, New York, N.Y.). M serotypes 2, 8, and 12 were obtained from the Center for Disease Control, Atlanta, Ga. Group C *Streptococcus* type 26RP66 (ATCC 21597) and phage C1 were obtained from V. Fischetti (Rockefeller University, New York, N.Y.). Bacterial cultures were maintained on blood agar slants containing 5% sheep erythrocytes and kept virulent by mouse passage. Fifty percent lethal dose (LD_{50}) values were as follows: type 2, 1.05×10^3; type 8, 3×10^3; type 12, 1×10^3 and type 14S23, 1×10^3. Phage C1 was maintained in sterile Todd-Hewitt broth (Difco Laboratories, Detroit, Mich.).

Growth of organisms. *S. pyogenes* 14S23 was grown on blood agar slants for 24 h at 37°C. Stock cultures were inoculated into Todd-Hewitt broth supplemented with 2% yeast extract (Difco) and grown overnight. These cultures were used to inoculate 16 liters of Todd-Hewitt broth supplemented with 2%

yeast extract. After 1 h of incubation, glucose was added to a final concentration of 2%. The pH was maintained at 7.8 by addition of 5 N NaOH. After 8 h of incubation at 37°C, cells were harvested with a Sorvall SZ-14 continuous-flow rotor (Ivan Sorvall Inc., Newtown, Conn.) at 25,000 × *g*. The cell pellet was washed three times in 0.02 M phosphate buffer, pH 7.2, containing 0.01 M $MgCl_2$ (PMB) to remove traces of growth medium.

Group C *Streptococcus* 26RP66 was grown in Todd-Hewitt broth overnight at 37°C for lysate production.

Production and partial purification of PAL. Phage-associated lysin (PAL) was produced and purified by a modification of the method of Fischetti et al. (8). Three liters of phage C1 lysate, at a titer of 5 × 10^9 plaque-forming units per ml, was stored for 4 weeks at 4°C to inactivate PAL. The lysate was then heated to 37°C and inoculated with 1 liter of an overnight culture of group C streptococci 26RP66. Growth was allowed to continue at 37°C for 25 min, and the culture was placed in an ice bath. Cells were centrifuged at 14,900 × *g*. The cell pellet was resuspended in 60 ml of 0.05 M KPO_4 buffer, pH 6.1, containing 5 × 10^{-4} M dithiothreitol (Sigma Chemical Co., St. Louis, Mo.) and 5 μg of deoxyribonuclease (Sigma). This suspension was incubated at 37°C until lysis of the cells was complete. Ethylenediaminetetraacetic acid was added to a final concentration of 0.005 M. Cell debris was removed by centrifugation at 27,000 × *g* for 2 h, and the pellet was discarded. Disodium tetrathionate (Pierce Chemical Co., Rockford, Ill.) was added in the cold (4°C) to a concentration of 0.3 M, and the solution was stirred gently for 1 h. Dry ammonium sulfate was added to 50% saturation, and the solution was allowed to stand 18 h at 4°C. The sediment was collected by centrifugation at 27,000 × *g* for 1 h and resuspended in 50 ml of 0.05 M KPO_4 buffer (pH 6.1) containing 0.005 M ethylenediaminetetraacetic acid and dialyzed against 20 liters of this same buffer. The fluid in the dialysis bag was removed and centrifuged at 36,000 rpm in a Beckman 60 Ti rotor for 3 h to remove phage particles. The supernatant fluid was stored at −51°C.

Partial purification of PAL was accomplished with a cellulose-phosphate ion-exchange gel (Sigma) equilibrated with 0.1 M KPO_4 buffer, pH 6.1, containing 0.005 M ethylenediaminetetraacetic acid and 10% glycerol. Before application to the column, crude PAL was dialyzed overnight against 10 volumes of the starting buffer. A 20-ml amount of crude enzyme was applied to the column. The PAL was eluted with running buffer containing 0.4 M NaCl. The protein peak containing the PAL was pooled, dialyzed against 20 volumes of starting buffer, and stored frozen at −51°C. Average activity as measured by the procedure of Fischetti et al. (8) was 20,000 U/ml.

Isolation of ribosomes. (i) PalR. For PAL-isolated ribosomes (PalR), *S. pyogenes* 14S23 was disrupted with the partially purified PAL as follows. Frozen cells (90 g) were washed twice and suspended at a ratio of 0.5 ml of buffer per g of cells in 0.05 M KPO_4 buffer, pH 6.1, with 0.005 M ethylenediaminetetraacetic acid. A 15-ml amount of partially purified PAL was reactivated with 0.1 M dithiothreitol. Reactivated enzyme was mixed with the cell suspension, and the mixture was incubated at 37°C for 1 h. Two additional portions of reactivated PAL were similarly added to the mixture at 1-h intervals. After 3 h, lysis of cells was determined by Gram stain. After lysis was complete, 10 mM $MgCl_2$ was added, and the incubation mixture was centrifuged at 27,000 × *g* for 25 min to remove whole cells and cell debris. The pellet (P-1) was stored at −51°C. The supernatant fluid was centrifuged at 56,000 rpm in a Beckman 60 Ti rotor for 3 h. The resulting ribosome pellet was washed 2× with PMB containing 1 M NH_4Cl, the washings were saved, and the pellet was stored at −51°C.

(ii) BR. Bead-isolated ribosomes (BR) were isolated by a modification of the method of Misfeldt and Johnson (23). All procedures were conducted at 4°C. The washed, packed cells were resuspended in 1 volume of PMB. Lysozyme hydrochloride (Sigma) was added in 15 mg/g of packed cells. After incubation for 1 h at 37°C, 2 μg of deoxyribonuclease (Sigma) per ml was added, and the slurry was divided into 25-ml portions. Each portion was added to a stainless steel flask containing 30 g of 0.017-mm glass beads. Cells were disrupted by shaking for four 3-min periods in a Braun MSK homogenizer. Cells were kept cool by circulating liquid CO_2 through the vibrating chamber. The cell extract was filtered through a sintered glass funnel to remove the glass beads. The filtrate was centrifuged at 27,000 × *g* for 30 min to remove whole cells and cell debris. The supernatant fluid was removed and passed through a 0.45-μm filter membrane (Millipore Corp., Bedford, Mass.) with positive pressure. Ribosomes were pelleted by ultracentrifugation at 125,000 × *g* for 3 h. The ribosomal pellet was washed in PMB containing 1 M NH_4Cl and stored frozen at −51°C. Washings were saved, pooled, and lyophilized.

Immunization and challenge. All mice were immunized subcutaneously with 310 μg of total protein emulsified in incomplete Freund adjuvant (GIBCO Laboratories, Grand Island, N.Y.) as described by Schalla and Johnson (28). Mice were challenged 1 week after the last immunization with homologous and heterologous M types.

Preparation of radioiodinated cells. *S. pyogenes* 14S23 was surface radiolabeled with ^{125}I by the lactoperoxidase technique as described by Vitteta et al. (34). Frozen cells (30 g) were washed twice in PMB. Cells were resuspended in 20 ml of PMB with a Tri-R stirrer (Tri-R Instruments, Inc., Jamaica, N.Y.). A 6-mg amount of lactoperoxidase (Sigma) in 2 ml of distilled water was added. A 5-mCi quantity of carrier-free $Na^{125}I$ (Amersham Inc., Arlington, Heights, Ill.) and 750 μl of 0.03% H_2O_2 were added. The reaction mixture was incubated at room temperature for 1.5 h, with 750 μl of 0.03% H_2O_2 added every 4 min. The reaction was stopped by addition of 50 ml of cold 50 mM cysteine-hydrochloride, pH 7.4. Cells were pelleted by centrifugation at 27,000 × *g* and washed once more in cold cysteine-hydrochloride. The cells were washed three times in 0.05 M KPO_4 buffer, pH 7.2, checked for morphology by Gram stain, and frozen at −51°C.

Isolation of ^{125}I cell wall material. Cell wall material from the iodinated cells was prepared as follows. Pellet P-1 from the enzymatic disruption of radioiodinated cells was resuspended in 1 volume of

PMB containing 2% sucrose. Three milliliters of this suspension was layered on top of a 30-50-70% stepwise sucrose density gradient in 0.05 M phosphate buffer. After centrifugation at 8,000 rpm in a Beckman L5-65 ultracentrifuge (SW27.1 rotor) for 15 min, fractions of 1 ml were collected from the top of the tubes. Fractions were counted in a Nuclear-Chicago 132B γ counter, and the bands containing radioactivity were pooled. Absence of whole cells was determined by Gram stain.

Polyacrylamide electrophoresis. Proteins were examined in polyacrylamide gels by the discontinuous system of Laemmli (17).

Autoradiography. Polyacrylamide gels were dried with a Hoeffer slab gel drier. Autoradiograms were generated by exposure of dried gels to Kodak X-Omat XR-5 X-ray film for 24 to 48 h.

Immunodiffusion. Immunodiffusion analysis was carried out by the method of Ouchterlony (26) in 0.1 M Veronal-buffered 1% agarose containing 3% polyethylene glycol 6000.

Biochemical assays. Protein was determined by the method of Lowry et al. (21). Ribonucleic acid (RNA) was measured by the orcinol method (3). Methyl pentose was determined by colorimetric analysis (14). Bovine serum albumin (Sigma), yeast RNA (Sigma), and L(+)-rhamnose (Sigma) served as standards.

Statistical evaluation. The level of significance for protection was determined by the chi-square method of Fisher and Yates (9).

RESULTS

Analysis of partially purified PAL. Partially purified PAL was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and immunodiffusion analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis showed three protein bands. Partially purified PAL did not react with antisera to BR.

Chemical studies. Chemical assays indicated an RNA-to-protein ratio of 2:1 for BR, which agrees with previously reported values for these ribosomes (28). An RNA-to-protein ratio of 1:1 was obtained with the PalR. Methyl pentose determination showed that a small amount of C carbohydrate was present in both BR and PalR preparations. The C carbohydrate was removed from the ribosomes by washing with 1 M NH_4Cl.

Comparison of the immunogenicity of BR and PalR. The ability of both PalR and BR ribosome preparations to protect mice against challenge with the homologous M type was studied. The results are shown in Table 1. Both PalR and BR gave significant levels of protection in mice against challenge with 1,000 LD_{50} of the homologous M type. The protection observed in mice immunized with PalR was always lower (65 versus 94%) than the protection observed in mice immunized with BR when given the same amount of protein (310 μg).

Electrophoretic comparison of BR and PalR. Sodium dodecyl sulfate-polyacrylamide gels were run to compare proteins present in BR and PalR. The results are shown in Fig. 1. BR show a larger number of bands and more bands in the high-molecular-weight region than PalR. Each well had 65 μg of protein applied. A glycoprotein stain of the gel showed one band that migrated with the dye marker in each preparation.

Contamination of ribosomes with cell wall proteins. To determine whether cell wall proteins were contaminating the ribosomes, ribosomes and cell walls were isolated from washed radioiodinated cells. An electropherogram and autoradiograph of ribosomes and cell walls are shown in Fig. 2. All ribosome preparations showed contamination with radiolabeled proteins that migrate with radiolabeled cell wall

TABLE 1. *Protective ability of BR and PalR when challenged with the homologous M type*

Ribosome	Alive/total[a]	% Survival[b]
BR	16/17	94
PalR	13/20	65
Control	0/20	0

[a] All animals challenged with 1,000 LD_{50} of homologous M type.

[b] $P \leq 0.01$; significantly different from sham-immunized controls.

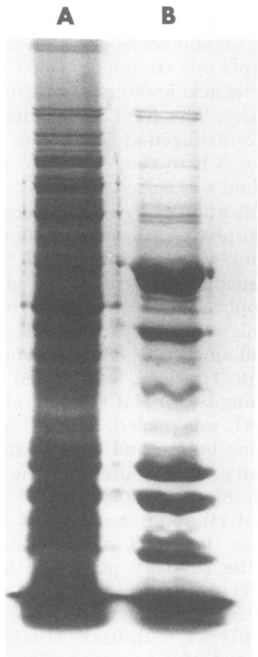

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electropherogram of NH_4Cl-washed BR and PalR (65 μg of protein applied to each well). (A) BR; (B) PalR.*

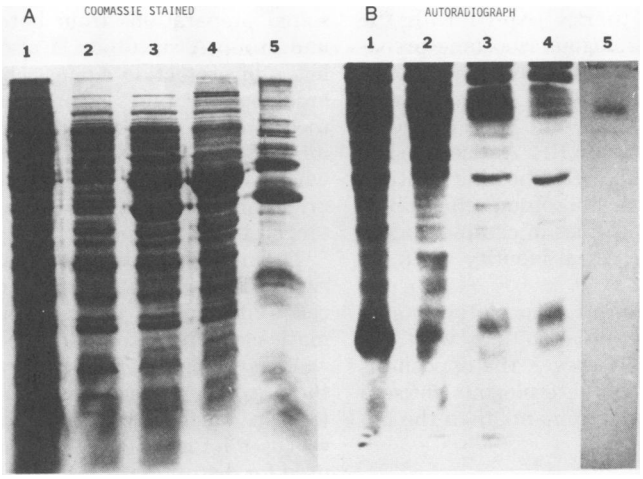

FIG. 2. *Sodium dodecyl sulfate-polyacrylamide gel electropherogram and autoradiograph of BR and PalR fractions. Column 1, unwashed BR; column 2, S. pyogenes cell walls after PAL treatment; column 3, unwashed PalR; column 4, NH_4Cl washed PalR; column 5, pooled washings of PalR.*

proteins. Washing of PalR with 1 M NH_4Cl removed many proteins. These proteins were recovered in the washings (Fig. 2A, column 5). However, even after washing 3× with 1 M NH_4Cl, some labeled proteins remained on the PalR (Fig. 2B, column 4). Although both PalR and BR contained labeled proteins, BR contained labeled proteins which were not found on the PalR.

Heterologous challenge. BR have been shown to protect mice against challenge with heterologous M types (28). The results of the heterologous challenge in mice immunized with either BR or PalR are shown in Table 2. BR isolated from M type 14 gave significant levels of protection in mice challenged with 100 LD_{50} of M types 2, 8, and 12. Mice immunized with PalR isolated from M type 14 also showed a significant level of protection when challenged with 100 LD_{50} of the homologous M type. However, when challenged with M types 2, 8, and 12 mice immunized with PalR failed to show any significant levels of protection against challenge with any of the heterologous M types.

Immunodiffusion studies. The results of immunodiffusion studies with BR and PalR are presented in Fig. 3. Figure 3A shows the antigenic relatedness of unwashed PalR, washed PalR, washed BR, and the NH_4Cl washings. Antisera to washed BR (Ab-B) reacts with the NH_4Cl washings, giving lines of complete identity with both BR and PalR. Lines of partial identity are observed between BR and washed PalR, demonstrating that antigenic determinants are found on BR which are not found on washed PalR. Washed PalR contain fewer precipitin lines than unwashed PalR, showing that some antigenic determinants are washed off in 1 M NH_4Cl. The second immunodiffusion slide (Fig. 3B) shows the reactions of antisera to PalR

TABLE 2. *Protective ability of BR and PalR when challenged with homologous and heterologous M types*

Challenge organism[a]	% Survival when immunized with	
	BR	PalR
Type 2	75 ($P < 0.01$)[b]	10 ($P > 0.01$)
Type 8	70 ($P < 0.01$)	11 ($P > 0.01$)
Type 12	75 ($P < 0.01$)	20 ($P > 0.01$)
Type 14	87.5 ($P < 0.01$)	65 ($P < 0.01$)

[a] Animals challenged with 100 LD_{50} of heterologous M type and 500 LD_{50} of homologous M type.

[b] *P* values are for experimental group versus sham-immunized controls.

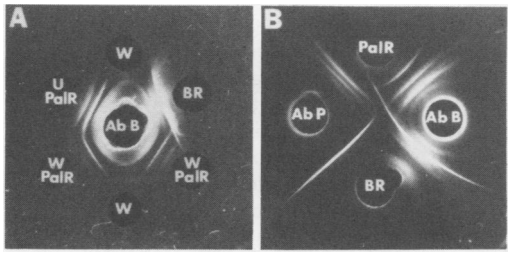

FIG. 3. *Immunodiffusion analysis of streptococcal ribosome fractions. (A) Antigenic relatedness of BR, PalR, and NH_4Cl washings. W, NH_4Cl washings from PalR; WPalR, NH_4Cl washed PalR; UPalR, unwashed PalR; Ab-B, antisera to washed BR. (B) Reactions of BR and PalR with homologous and heterologous antisera. Ab-P, antisera to washed PalR; Ab-B, antisera to washed BR; BR, NH_4Cl-washed BR; PalR, NH_4Cl-washed PalR.*

(Ab-P) and antisera to BR (Ab-B) with the homologous and heterologous ribosome preparations. Antisera to PalR show three precipitin lines with PalR, two of which show lines of complete identity with BR. The third antigenic determinant is not found on BR. Antisera to BR react with both PalR and BR, showing multiple determinants with both ribosome preparations. The BR and PalR show lines of complete identity, nonidentity, and partial identity when reacted with Ab-B.

The BR show more antigenic determinants with the homologous antisera than with the heterologous antisera. However, the opposite is true with the PalR. The heterologous antisera show more antigenic determinants than the homologous antisera.

DISCUSSION

Protective ribosomal fractions have been isolated from many gram-positive (22, 28, 32, 35) and gram-negative (2, 4, 11–13, 19, 29, 32) bacteria. However, the exact chemical nature of the protective antigen is unknown. The protective immunogenic moiety has been reported to be double-stranded RNA in ribosomes isolated from *M. tuberculosis* (36–38), a protein in the ribosomal fraction of *S. pneumoniae* (30), either lipopolysaccharide (4, 5), a protein attached to the ribosome (25), or an RNA-protein complex (29) in *Salmonella typhimurium*, and either a ribosomal antigen or a lipopolysaccharide-RNA complex in *Pseudomonas aeruginosa* (18, 20).

Investigators working with gram-negative organisms have shown that ribosome preparations may be contaminated with cell wall and cell membrane components. Eisenstein (4) has shown that ribosomes isolated from *S. typhimurium* are contaminated with lipopolysaccharide containing the O antigens expressed on the cell surface. However, Misfeldt and Johnson (24) showed that endotoxin contaminating protective ribosomal vaccines isolated from *S. typhimurium* did not totally account for their effectiveness. Lieberman (18, 20) has shown that a protective ribosomal fraction isolated from *P. aeruginosa* contains lipopolysaccharide as a contaminant. Pneumococcal ribosomes have also been shown to be contaminated with cell wall proteins (Swendsen and Johnson, unpublished data).

Swendsen and Johnson found that a protein extract of ribosomes isolated from *S. pneumoniae* gave significant levels of protection in mice (30). Schalla and Johnson, using *S. pyogenes* ribosomes, demonstrated that protein was the effective immunogen in this system (28). Ribosomal preparations from both *S. pneumoniae* and group A streptococci also gave significant levels of protection against challenge with heterologous serotypes of pneumococci and streptococci along with classical type-specific immunity (28, 30). However, it was never ascertained whether the protein antigens were of ribosomal origin or whether they were material which attached to the ribosomes during purification.

To investigate the possibility of producing ribosomal preparations free of contaminating cell wall material, it was decided to use enzymatic cleavage of the group A streptococcal cell wall to disrupt the cells. This is in contrast to the previous procedure of Schalla and Johnson (28), in which physical disruption of the cells was used for isolation of ribosomes. The enzyme used for disruption was PAL, which is produced by certain group C streptococci when infected with phage C1. The enzyme is produced in excess and is released into the extracellular fluid upon lysis of the cells. PAL exhibits an extremely narrow substrate range, acting as an endoacetyl hexosaminidase (1) on cell walls of only groups A, C, and E streptococci (6). PAL has been shown to release M protein, C carbohydrate, and other cell surface antigens (15, 16). It was therefore felt that PAL disruption of *S. pyogenes* might yield ribosomes free of the contaminating cell surface antigens. However, the presence of labeled bands in the autoradiograph (Fig. 2) of ribosomes isolated by physical and chemical disruption suggests that cell wall proteins are present in both PalR and BR ribosomal preparations, since the lactoperoxidase technique for radioiodination of cell proteins has been shown by Phillips and Morrison (29) to label only surface membrane proteins in intact mammalian cells. The great majority of labeled proteins were located in the high-molecular-weight region. Although some of the labeled protein bands are removed by washing in 1 M NH_4Cl, many appear to be tightly bound to the ribosomes (Fig. 2B, columns 3 and 4). Since these proteins are bound to the ribosomes, they may be exposed to the mouse immune system and be antigenic.

The data presented in heterologous challenge experiments with BR are consistent with the earlier work of Schalla and Johnson (28). The BR preparations again gave significant levels of protection to challenge with the heterologous M types. However, when mice immunized with PalR were challenged with heterologous and homologous M types, only the mice challenged with the homologous M type were significantly protected. This indicates that enzymatic cleavage of the cell wall may in some way alter or eliminate an antigenic determinant responsible

for heterologous protection. Thus, there may be two antigenic determinants which are responsible for the protection in BR, one for homologous protection and one for heterologous protection.

The immunodiffusion studies suggest that although some antigenic determinants are washed off of the PalR, some of these determinants remain on the PalR and are identical to ones on the BR. In addition, the lines of partial identity observed between BR and PalR suggest that a piece of cell wall material contaminating the ribosomes may be partially degraded by the PAL. Although there are antigenic determinants found on PalR which are not found on BR (Fig. 3B), it is apparent from the number of determinants recognized by the homologous sera that the BR act as a much better immunogen than the PalR. It is possible that the PAL has changed some determinants on the cell surface such that when they are bound to the ribosomes, they are not immunogenic in the mouse. However, these antigenic determinants may still be accessible to precipitating antibodies. If this were the case, then it could explain why the heterologous system (Ab-B and PalR) detects more determinants than the homologous system (Ab-PalR and PalR) (Fig. 3B).

Further studies on the isolation and identification of the protective immunogenic moiety may lead to an understanding of the mechanism by which ribosomal vaccines of *S. pyogenes* elicit protective immunity against homologous and heterologous challenge.

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