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BRIEF REPORT

Detection of opsonic antibodies against *Enterococcus faecalis* cell wall carbohydrates in immune globulin preparations

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Abstract Three different commercially available polyvalent immune globulins (IG) were investigated for the existence of antibodies against cell wall carbohydrates of four different *E. faecalis* serotypes (using a cell wall carbohydrate-enzyme-linked immunosorbent assay), and whether these antibodies mediated opsonic killing (using an opsonic-killing assay). All three IG preparations contained antibodies against all four serotypes (CPS-A to CPS-D). However, only one of the three IG preparations showed opsonic killing against all four serotypes. Average killing was higher against serotypes A and B (72 and 79 %, respectively) than against serotypes C and D (30 and 37 %, respectively). Such IG preparations could play a role as an adjuvant therapeutic option in life-threatening infections with *E. faecalis*, particularly when resistant strains are involved.

Keywords *E. faecalis* · Enterococci · Immune globulins · Immunotherapy · Cell wall carbohydrates · Opsonic antibodies

Introduction

Although enterococci usually are considered facultative pathogens with low virulence, they also can cause a variety of infections, especially in immunosuppressed patients in hospital settings [1]. The clinical outcome in these patients may be compromised by resistance to conventional therapeutic regimens [1]. Therefore, there is an urgent need to identify novel therapeutic and prophylactic options to combat enterococcal infections, one such option being immunotherapy. In an experimental model of systemic enterococcal infections, mice were protected when they

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received antibodies against enterococcal cell wall carbohydrates [2, 3]. The cell wall of enterococci consists of different cell wall carbohydrates and glycoconjugates [4]. Among these carbohydrate-containing antigens, lipoteichoic acid (LTA) and capsular polysaccharides play a key role in the protection of enterococci against neutrophil-mediated killing [3–5]. Our group has studied the diversity of *Enterococcus faecalis* cell wall carbohydrates using four type-specific immune sera raised against carbohydrates which had been extracted from the cell walls of four *E. faecalis* prototype strains (CPS-A to CPS-D) [6]. The main target of opsonic antibodies against CPS-A and CPS-B strains was determined to be LTA [3]. The *cps* locus codes for genes involved in the production of capsular polysaccharide antigens [5, 7]—antigens whose chemical structures have yet to be fully determined. The *cps* locus consists of 11 genes (named *cpsA* to *cpsK*) [5–7]. In CPS-A and -B strains only *cpsA* and *cpsB* genes are detected, whereas in CPS-C and -D strains *cpsA* to *cpsE* and *cpsG* to *cpsK* genes are present [5–7]. The *cpsF* gene is variably expressed in CPS-C or -D strains [5–7]. Our group was able to demonstrate that in CPS-A and CPS-B strains, LTA is surface exposed and seems not to be masked by a polysaccharide capsule [4]. In contrast, CPS-C and CPS-D strains possess a polysaccharide capsule, which consists of a diheteroglycan [4]. Immune sera raised against capsular polysaccharide were protective in a mouse bacteremia model against CPS-C and CPS-D strains [4]. A monoclonal antibody against enterococcal LTA is currently not available. We therefore designed the current study to test whether three commercially available polyvalent immune globulin (IG) preparations contained opsonic antibodies against the four *E. faecalis* CPS strains, and to test whether IG preparations could offer a potential adjuvant treatment option for life-threatening enterococcal infections. We chose to study IG treatment in *E. faecalis* as a model for its potential use against *E. faecium,* because the latter is more likely to develop antibiotic resistance. For this reason, additional treatment options are needed.

Materials and methods

Bacterial strains and cultures

Bacterial strains used in the study are described in Table 1. All *E. faecalis* strains were grown without agitation at 37 °C, either in tryptic soy broth (TSB, Becton-Dickinson, Sparks, MD, USA), or, for the cell wall carbohydrate (CWC)-ELISA, in Columbia broth (CB, Difco Laboratories, Detroit, MI, USA), with the addition of 0.5 % glucose.

Table 1 *Enterococcus faecalis* prototype strains and immune sera used in the present study

Strain	Reference	Source	Immune serum
CPS-A	Prototype strain [6] (*E. faecalis* 12030)	Clinical isolate, USA	Anti-CPS-A [6]
CPS-B	Prototype strain [6] (*E. faecalis* 12107)	Clinical isolate, USA	Anti-CPS-B [6]
CPS-C	Prototype strain [6] (*E. faecalis* type 2)	Prototype Japanese strain	Anti-CPS-C [6]
CPS-D	Prototype strain [6] (*E. faecalis* type 5)	Prototype Japanese strain	Anti-CPS-D [6]

Immune globulins and sera

Three different, commercially available pooled polyvalent IG preparations (IG1 (Gamunex® 10 %, Talecris Biotherapeutics, Frankfurt, Germany), IG2 (Octagam®, Octapharma, Langenfeld, Germany) and IG3 (Beriglobin®, CSL Behring, Marburg, Germany) were used. For a proof-of-concept study, all experiments initially were performed with one batch of each IG preparation. Positive findings were confirmed using a second, different batch of IG preparations. For the CWC-ELISA, a control serum from a healthy human volunteer with known quantities of antibodies against all four *E. faecalis* prototype strains was employed [8]. For opsonophagocytic killing assays, immune rabbit sera (IRS) against all four *E. faecalis* prototype strains were used (Table 1) [6]. Immune sera were heated at 56 °C for 30 min to inactivate complement. Absorptions of sera with the homologous *E. faecalis* strain were performed as described elsewhere [8].

Quantitative determination of cell wall carbohydrate-specific antibodies by ELISA (CWC-ELISA)

The assay was performed as described elsewhere with some minor modifications regarding the initial culture volume [6, 8]. The carbohydrate content of the cell wall extracts was measured by hexose assay and was adjusted to a final concentration of 15 μg carbohydrate per ml [8]. The extracts were divided in aliquots and stored at −80 °C. As a blocking agent in the ELISA, 3 % skim milk in phosphate-buffered saline was used. IG were applied (unabsorbed or absorbed with the homologous *E. faecalis* strain) in dilutions of 1:100, 1:500, 1:2,500 and 1:5,000. A human immune serum was only used in a single 1:1,000 dilution as a positive control. Each strain was tested at least in duplicate. Both positive (i.e., human immune serum) and negative controls (i.e., no IG or no human immune serum) were included on all plates.

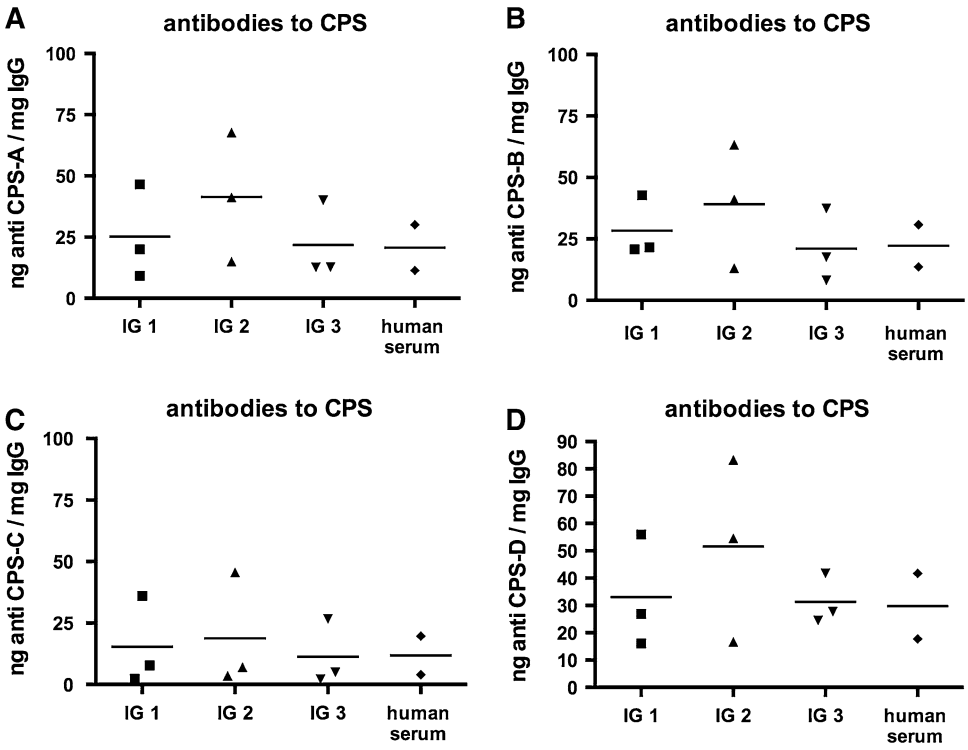

Fig. 1 IgG concentrations against cell wall carbohydrates of four *E. faecalis* prototype strains (CPS-A, CPS-B, CPS-C, CPS-D) in three immune globulin (IG) preparations compared to a human immune serum detected by a CWC-ELISA. Results represent means of duplicates of two (for human sera) or three (for IG preparations) independent experiments. *Horizontal lines* show the mean antibody concentrations of all independent experiments that were performed

Resulting titers at an optical density of 405 nm were normalized against negative controls. Lastly, antibody concentrations were calculated by linear regression using the respective IgG standard [8].

In vitro opsonophagocytosis assay (OPA)

IG preparations were tested for their opsonic killing activity against the four *E. faecalis* prototype strains. The opsonophagocytic assay was performed as described elsewhere with some minor modifications [6]. IG preparations were used both undiluted and 1:50 diluted either unabsorbed or absorbed with the homologous *E. faecalis* strain. Unabsorbed or absorbed immune rabbit sera served as a positive control in a 1:50 dilution.

Statistical analysis

For statistical analysis, Microsoft Excel and GraphPad PRISM V.6 were used. Correlations were calculated using linear regression models and comparisons between two groups were performed with Student's *t* test. A p value of <0.05 was considered to be statistically significant.

Results and discussion

Using the CWC-ELISA, IgG antibodies against cell wall carbohydrates of all four *E. faecalis* prototype strains were detected in all three IG preparations (Fig. 1). Corrected for the IgG content of the three IG preparations (i.e., measured in ng/mg IgG), CPS-specific IgG concentrations were found to be within the same range against all four prototype strains, (i.e., 9–70 ng/mg CPS-specific IgG for CPS-A, 7–60 ng/mg CPS-specific IgG for CPS-B, 5–50 ng/mg CPS-specific IgG for CPS-C, and 14–92 ng/mg CPS-specific IgG for CPS-D, respectively). The lowest CPS-specific IgG concentration in all three IG preparations was detected against CPS-C. The highest CPS-specific IgG concentrations against all four prototype strains consistently were identified in the IG2 preparation.

Using the *opsonophagocytic killing assay*, opsonic antibodies against all four *E. faecalis* prototype strains were detected in only one IG preparation (i.e., IG2; Fig. 2). Killing of all four prototype strains was achieved only when the IG2 preparation was used undiluted. Statistically, this killing was significantly different both for IG2 and for the respective immune rabbit sera from a negative control for all four prototype strains (Table 2). Killing by IG2 was higher against CPS-A and CPS-B (i.e., 72 and 79 %, respectively) than against CPS-C and CPS-D (i.e., 30 and 34 %, respectively). Some killing (i.e., 38 %) was also seen when using a diluted IG2 preparation (i.e., 1:50) against CPS-A, but not against the other three prototype strains. The killing of CPS-A by diluted IG2 was statistically not different from killing by diluted IG2 (data not shown). In addition, some reduction in viable counts (i.e., 11 %) was also noted for diluted IG2 preparation against

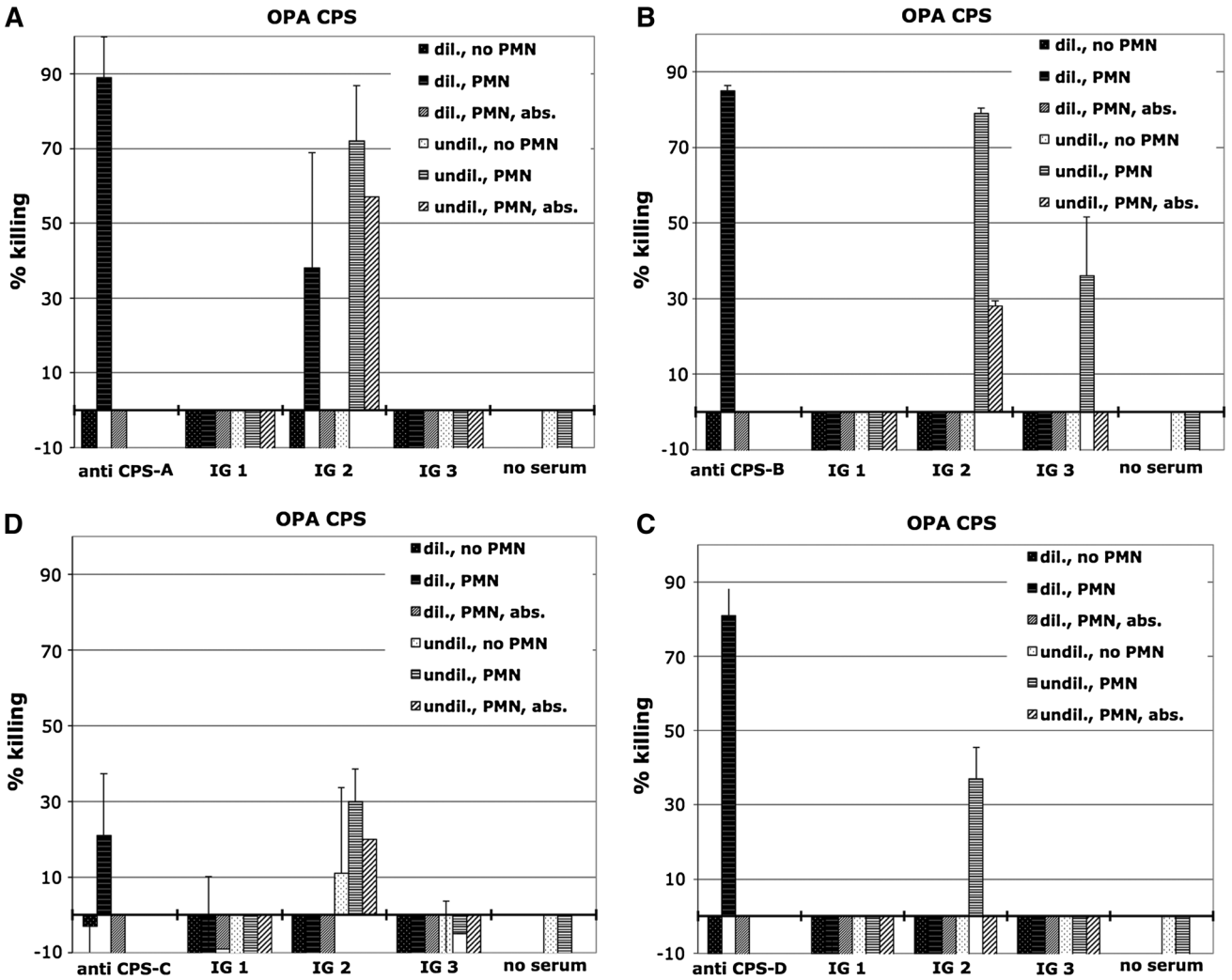

Fig. 2 Opsonophagocytic killing of three immune globulin (IG) preparations compared to respective immune rabbit sera (anti-CPS-A to anti-CPS-D) against four *E. faecalis* prototype strains (CPS-A, CPS-B, CPS-C, CPS-D). Negative killing indicates growth of bacteria during the 90-min incubation of the assay. *Bars* represent the mean killing and error bars the standard deviation of a representative assay performed in duplicates. *OPA* opsonophagocytosis assay, *dil.* diluted (i.e., 1:50), *undil.* undiluted, *PMN* polymorphonuclear neutrophils, *abs.* absorbed with the homolog enterococcal strain

CPS-C, even in the absence of leukocytes. This reduction in viable counts was statistically not different from killing observed in the presence of antibodies, complement and leukocytes [$p = 0.124$ (95 % CI −50.8 to 12.8)]. We twice repeated the assay—comparing diluted IG2 with and without leukocytes—and no killing without leukocytes was detected (data not shown). For these repeat assays, two different batches of IG2 preparations were used. Absorption of diluted immune sera or IG2 resulted in complete (i.e., against CPS-B and -D) or reduced killing activity (i.e., against CPS-A and -C), respectively (Table 3). The degree of reduction in killing was dependent upon whether IG preparations were used in diluted or undiluted form, as well as upon the degree of dilution. The latter was tested with IG2 against CPS-A (Fig. 3). Killing of undiluted IG in combination with neutrophils and complement was only partially reduced by absorption, whereas in dilutions of ≥1:20, killing was no longer detectable (Fig. 3). The same was seen for absorption of immune sera in a 1:50 dilution. The differences in the absorption studies of undiluted IG2 against CPS-A in Fig. 2 (i.e., complete abolition of killing by absorption) vs. Fig. 3 (i.e., no reduction of killing by absorption) might be explained by the fact that two different batches of IG2 were used in the two experiments. No significant absorption effect was noted in CPS-C, mainly because, due to the presence of a capsule, this strain is known to be more resistant against opsonic killing [4].

Taken the assay results together, all three polyvalent IG preparations contained antibodies that bound to cell wall carbohydrates of four *E. faecalis* prototype strains.

Table 2 Comparison of opsonophagocytic killing by either IG2 preparation or by the respective immune rabbit sera (anti-CPS-A to anti-CPS-D) vs. no sera against four *E. faecalis* prototype strains (CPS-A to CPS-D)

Strain	IG2 preparation or immune rabbit serum	Mean % killing by IG2 or immune rabbit serum (SD)	Mean % killing without serum (SD)	*p* (*t* test) and 95 % CI
CPS-A	IG2 (undil.)	71.5 (6.4)	−189.0 (59.4)	0.0253 (−442.2 to −78.8)
	Anti-CPS-A (dil.)	87.8 (9.9)		0.0005 (−351.1 to −202.4)
CPS-B	IG2 (undil.)	79.0 (1.4)	−109.5 (53.0)	0.0374 (−349.9 to −27.1)
	Anti-CPS-B (dil.)	85.0 (1.4)		0.0352 (−355.9 to −33.1)
CPS-C	IG2 (undil.)	29.5 (5.0)	−26.5 (0.7)	0.004 (−71.2 to −40.8)
	Anti-CPS-C (dil.)	21.0 (7.1)		0.011 (−69.4 to −25.8)
CPS-D	IG2 (undil.)	37.0 (31.1)	−120.0 (15.6)	0.0237 (−262.8 to −51.2)
	Anti-CPS-D (dil.)	81.0 (7.1)		0.0036 (−253.0 to −149.0)

Negative killing indicates growth of bacteria during the 90-min incubation of the assay

SD standard deviation, *95 % CI* 95 % confidence interval, *dil.* diluted (i.e., 1:50), *undil.* undiluted

Table 3 Comparison of opsonophagocytic killing by undiluted IG2 preparation vs. undiluted and absorbed IG2 preparation against four *E. faecalis* prototype strains (CPS-A to CPS-D)

Strain	Mean % killing by undiluted IG2 (SD)	Mean % killing by undiluted and absorbed IG2 (SD)	*p* (*t* test) and 95 % CI
CPS-A	71.5 (6.4)	56.5 (14.9)	n.s. (−64.2 to 34.2)
CPS-B	79.0 (1.4)	28.0 (1.4)	0.0008 (−57.1 to -44.9)
CPS-C	29.5 (5.0)	19.5 (5.0)	n.s. (−31.3 to 11.3)
CPS-D	37.0 (31.1)	−48.5 (23.3)	0.0251 (−150.7 to -20.3)

Negative killing indicates growth of bacteria during the 90-min incubation of the assay

SD standard deviation, *95 % CI* 95 % confidence interval, *n.s.* non-significant

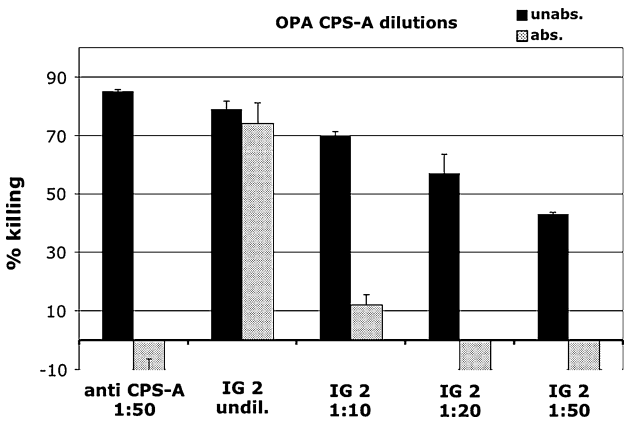

Fig. 3 Opsonophagocytic killing of undiluted vs. diluted immune globulin preparation (i.e., IG2) compared to the respective immune rabbit serum (i.e., anti-CPS-A) against *E. faecalis* prototype strain CPS-A. Negative killing indicates growth of bacteria during the 90-min incubation of the assay. *Bars* represent the mean killing, *error bars* the standard deviation of a representative assay performed in duplicates. *OPA* opsonophagocytosis assay, *undil.* undiluted, *unabs.* unabsorbed, *abs.* absorbed

However, only one IG preparation (i.e., IG2) showed neutrophil-mediated killing in an in vitro assay. Similar findings with respect to sera from healthy human volunteers previously have been reported by our group [8]. However, it should be noted that the negative findings of the immune globulin preparations IG1 and IG3 are based only on the fact that one batch each was used. It cannot be excluded that different batches may have shown different results.

The CWC-ELISA uncovers a broad range of antibodies that bind to a variety of cell wall antigens, whereas the OPA only detects antibodies that are opsonic in nature. This finding may be related to the TCA method used to extract carbohydrates from the cell wall for the CWS-ELISA. TCA extracts contain a mixture of carbohydrate antigens with protein levels below the detection limit of the modified Bradford assay [8]. The extracted cell wall carbohydrates do not contain only serotype-specific enterococcal LTA rather, they most likely also contain other common carbohydrates that anchor to the cell wall, such as tetraheteroglycans [4], which may reduce type specificity and induce cross-reactivity of the detected antibodies.

Based upon absorption studies (Figs. 2, 3), the killing in the opsonophagocytic assays—whether by IG preparations or by type-specific immune sera—was observed to be antibody specific. Whenever IG preparations or immune sera were absorbed with the homologous enterococcal strain, opsonic killing was reduced against all four prototype strains. The degree of reduction in killing was dependent upon whether IG preparations were used in diluted or undiluted form, as well as upon the degree of dilution (Fig. 3).

Of all four prototype strains, low opsonophagocytic killing against CPS-C was mediated by both the specific rabbit immune serum and by the IG2 preparation. This

indicates a higher resistance on the part of the particular prototype strain—a resistance which may be explained by the fact that this strain possesses a polysaccharide capsule that probably shields the type-specific LTA antigen [4]. Indeed, our group was able to show that both CPS-C and CPS-D prototype strains are encapsulated enterococcal strains that mask their LTA [4]. These strains showed resistance to killing by type-specific anti-LTA antibodies [3, 5]. However, killing of CPS-C and CPS-D was mediated by antibodies raised against purified capsule antigen [4]. In a mouse bacteremia model, this antiserum was found to be protective [4]. By contrast, anti-LTA antibodies were able to kill CPS-A and CPS-B strains, which indicates that these two prototype strains are likely to be unencapsulated [4].

Overall, we observed a modest killing effect by one IG preparation, at least when it was used in undiluted form. This finding may be explained by two limiting factors. First, the overall concentration of opsonic antibodies in IG preparations is likely to be quite low. Gagliani et al. have been able to demonstrate that the killing of sera from patients with hypogammaglobulinemia could be supplemented by substituting specific antibodies through IG; they further showed that the degree of killing was correlated to the antibody amount within the substituted IG [9]. Second, standard IG preparations lack IgM antibodies. However, opsonic killing seems to be more efficient by anti-enterococcal IgM antibodies than by IgG antibodies [8, 9]. For future studies, it would be interesting to compare IG preparations that contain both IgG and IgM antibodies.

Our findings support the hypothesis that certain IG preparations are potentially useful as adjuvant therapies in life-threatening *E. faecalis* infections, especially when resistant enterococci are involved. To date, similar experiments with *E. faecium* or with resistant enterococci (e.g., Vancomycin-resistant *E. faecium*) have not been performed. Binding and killing experiments against cell wall carbohydrates of *E. faecalis* were used as a model for other enterococcal infections, primarily because a serotyping scheme only exists for *E. faecalis* and not for *E. faecium*.

In principle, application of IG may be an additional therapeutic option in human infections. Extrapolating from the opsonophagocytic killing assay results, to achieve the same amount of killing in vivo vs. in vitro, 12.5 mg IG2 per ml human blood would be required. Therefore, in humans, 20 ml of the 5 % IG2 preparation per kg body weight would need to be infused intravenously. Administering this amount of IG in a clinical setting would be achievable. Although animal and clinical studies will be warranted to confirm this hypothesis, hyper-immune sera with a higher content of type-specific and opsonic antibodies or monoclonal antibodies should have a greater potential to mediate protection than standard immune globulin preparations.

Clinical studies on the use of immune globulin preparations, as well as of other preventative or therapeutic immunotherapies in human enterococcal infections have yet to be reported. However, experiences with passive immunization strategies can be derived from staphylococcal infections. Recently, results of a clinical phase 2 study of a human chimeric monoclonal antibody developed against lipoteichoic acid (Pagibaximab) in neonatal sepsis became available [10]. Although the trial failed to demonstrate a statistically significant activity of the monoclonal antibody in preventing staphylococcal sepsis as compared to placebo, in infants that had received the highest dose of pagibaximab, no staphylococcal or other Gram-positive infections were documented.

In conclusion, our study findings indicate that although IG preparations contain antibodies that bind specifically to cell wall carbohydrates of different *E. faecalis* prototype strains, only certain IG preparations will induce neutrophil-mediated killing. Therefore, certain IG preparations may play a role as an adjuvant therapy in life-threatening enterococcal infections. However, additional studies are needed before therapeutic or prophylactic use of immune globulins in enterococcal infections can be recommended.

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