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Aggregation and Binding Substances Enhance Pathogenicity in Rabbit Models of *Enterococcus faecalis* Endocarditis

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We investigated the importance of enterococcal aggregation substance (AS) and enterococcal binding substance (EBS) in rabbit models of *Enterococcus faecalis* cardiac infections. First, American Dutch belted rabbits were injected intraventricularly with 10^8 CFU and observed for 2 days. No clinical signs of illness developed in animals given AS^- EBS^- organisms, and all survived. All rabbits given AS^- EBS^+ organisms developed signs of illness, including significant pericardial inflammation, but only one of six died. All animals given AS^+ EBS^- organisms developed signs of illness, including pericardial inflammation, and survived. All rabbits given AS^+ EBS^+ organisms developed signs of illness and died. None of the rabbits receiving AS^+ EBS^+ organisms showed gross pericardial inflammation. The lethality and lack of inflammation are consistent with the presence of a superantigen. Rabbit and human lymphocytes were highly stimulated in vitro by cell extracts, but not cell-free culture fluids, of AS^+ EBS^+ organisms. In contrast, cell extracts from AS^- EBS^- organisms weakly stimulated lymphocyte proliferation. Culture fluids from human lymphocytes stimulated with AS^+/EBS^+ enterococci contained high levels of gamma interferon and tumor necrosis factor alpha (TNF-α) and TNF-β, which is consistent with functional stimulation of T-lymphocyte proliferation and macrophage activation. Subsequent experiments examined the abilities of the same strains to cause endocarditis in a catheterization model. New Zealand White rabbits underwent transaortic catheterization for 2 h, at which time catheters were removed and animals were injected with 2×10^9 CFU of test organisms. None of the animals given AS^- EBS^- organisms developed vegetations or showed autopsy evidence of tissue damage. Rabbits given AS^- EBS^+ or AS^+ EBS^- organisms developed small vegetations and had splenomegaly at autopsy. All rabbits given AS^+ EBS^+ organisms developed large vegetations and had splenomegaly and lung congestion at autopsy. Similar experiments that left catheters in place for 3 days revealed that all rabbits given AS^- EBS^- or AS^+ EBS^+ organisms developed vegetations, but animals given AS^+ EBS^+ organisms had larger vegetations and autopsy evidence of lung congestion. These experiments provide direct evidence that these two cell wall components play an important role in the pathogenesis of endocarditis as well as in conjugative plasmid transfer.

Recently, *Enterococcus faecalis* and other enterococci have become increasingly recognized as significant causes of nosocomial infections (23, 26, 27, 30). They are important causes of bacteremia, endocarditis, and urinary tract infections. These organisms are also important because of their increasing incidence of resistance to vancomycin and other antibiotics and because of the potential of transferring antibiotic resistance to other bacteria.

An important mechanism for horizontal transfer of antibiotic resistance in enterococci is pheromone-inducible conjugation (10, 12, 34). The expression of conjugative transfer functions of plasmids such as pCF10 (58 kb; encodes tetracycline resistance [12, 14]) and pAD1 (60 kb; encodes hemolysin and bacteriocin production [10, 31]) is induced by peptide pheromones produced by recipient cells (13, 31, 34). The conjugation gene products induced by pheromones include a cell surface adhesin, aggregation substance (AS). This protein mediates the formation of mating aggregates between donor and recipient cells by binding to a cognate ligand on the recipient cell, enterococcal binding substance (EBS) (13, 14). The *prgB* gene of pCF10 encodes the AS protein, Asc10 (25), whose nucleotide and amino acid sequences are highly similar to those of AS proteins encoded by other pheromone plasmids (19, 20). The genetics of EBS are complex, with multiple, unlinked insertion mutations required to generate an EBS-negative phenotype (5, 12, 32). Lipoteichoic acid (LTA) appears to be an important component of EBS (7, 15, 32).

Previous studies of the pathogenicity of *E. faecalis* have shown that hemolysin contributes to the virulence of the organism in animal models, including murine peritonitis, rabbit endophthalmitis, and rabbit endocarditis (9, 11, 21, 25, 26). In their study, Chow et al. (9) also showed that AS contributed significantly to the production of experimental endocarditis. Hemolysin and AS were associated with increased mortality, and AS was associated with increased vegetation weight.

AS proteins of *E. faecalis* are thought to be virulence factors in enterococcal infections by promoting binding to a variety of eukaryotic cell surfaces (21, 25, 26). AS expression may be induced in vivo by eukaryotic factors in serum (6). AS contains amino acid motifs, Arg-Gly-Asp-Ser and Arg-Gly-Asp-Val, which are found in fibronectin and other proteins and which mediate binding to eukaryotic cell adhesion molecules of the integrin superfamily (18, 21, 25). Soluble LTA inhibits aggre-

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gate formation and may function as EBS (26). LTA from *E. faecalis* has previously been shown to induce both interleukin 1β and tumor necrosis factor alpha (TNF-α) production from macrophages (7).

This study was undertaken to evaluate the role of both AS and EBS in two rabbit models of *E. faecalis* cardiac infections. Greater insight into the role of these two factors in virulence may lead to alternative methods of prophylaxis and treatment of resistant enterococcal infections. Our studies indicate that the presence of both cell surface components is associated with both increased mortality and formation of vegetations.

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MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in these studies were constructed and characterized previously (5, 26, 32). The EBS^- mutant INY3000 was generated by Tn*916* insertion in the *E. faecalis* 0G1SSp chromosome (32). Plasmid pINY1801 (26) contains a fragment of pCF10 cloned into shuttle vector pWM401 (35) and confers constitutive expression of Asc10.

This isogenic set of strains with various combinations of AS and EBS phenotypes was constructed several years ago (26, 32). The strains have maintained the appropriate phenotypes for over 10 years, and during the course of the present study, they were periodically checked with antibodies. Finally, the AS^+ EBS^+ strain self-aggregated in liquid culture, which is consistent with its phenotypes. Furthermore, the remaining organisms did not self-aggregate, but when they were cocultured in liquid, AS^+ EBS^- and AS^- EBS^+ organisms formed aggregates.

Intraventricular injection model of endocarditis. Bacteria were cultured to approximately 10^8 CFU/ml in a dialyzable beef heart medium (28). Estimations of cell density were determined by absorbancy at 620 nm and verified by plate counts. Organisms were injected in 1-ml volumes of beef heart medium directly into the left ventricles of American Dutch belted rabbits purchased from Birchwood Farms, Grantsburg, Wis. After termination of the experiment, all animals were evaluated for needle puncture sites to ensure that enterococci were injected into the left ventricle. Animals were examined for 2 days for signs of infection, including fever, diarrhea, clear amber urine, mottled faces, and death. Whether they succumbed to infection or were euthanized after the 2-day test period, all animals were examined for gross tissue effects due to infection. All major organ systems, except for the nervous system, were examined.

Transaortic catheterization models of endocarditis. New Zealand White rabbits (Birchwood Farms) were anesthetized with ketamine (25 mg/kg; Phoenix Pharmaceutical, Inc., St. Joseph, Mo.) and xylazine (20 mg/kg; Phoenix), and the left carotid arteries in their necks were exposed. Subsequently, catheters (outside diameter, 1.27 mm; Becton Dickinson, Sparks, Md.) were inserted until the tubing was adjacent to or transversed the aortic valve. In some experiments, catheters were left in place for 2 h and then removed (animals remained anesthetized for the entire 2-h period). In other experiments, catheters were implanted and remained in place for the entire 3-day test period. After catheterization, animals were closed with sutures and then injected intravenously with enterococci. Animals were monitored for 3 days and then their hearts and other organs were examined grossly for signs of infection. During these experiments, different catheter sizes were evaluated. Catheters of smaller diameters did not lead to cardiac vegetations in experiments where catheters were left in place for 2 h. Thus, we discontinued the use of these materials.

Bacteria were cultured to the indicated cell densities, as determined by absorbance at 620 nm, in a dialyzable beef heart medium (28). Cell numbers were verified by plate counts. Organisms were injected intravenously in 2-ml volumes of beef heart medium in the left marginal ear veins of test animals. The optimal cell numbers for use in these studies were previously determined by dose-response experiments. Animals (two per group) were given 2×10^7, 2×10^8, 2×10^9, 5×10^9, or 2×10^{10} AS^+ EBS^+ enterococci for dose-response determinations. No cardiac vegetations or other signs of infection were seen with a 2×10^7 dose, one of two animals developed small vegetations with a 2×10^8 dose, and both animals receiving a 2×10^9 dose developed numerous large vegetations and lung congestion. Finally, animals given either 5×10^9 or 2×10^{10} enterococci succumbed overnight without significant visible cardiac effects but with significant lung congestion.

Lymphocyte mitogencity. Rabbit splenocytes and human peripheral blood mononuclear cells were cultured in RPMI cell culture medium in quadruplicate on 96-well microtiter plates (2×10^5 lymphocytes/well/200 μl) (2). Cultures were incubated at 37°C in the presence of 7% CO_2 with serial 10-fold dilutions of enterococcal-cell extracts or supernatants. Cells incubated with 1 μg of toxic shock syndrome toxin-1 (TSST-1) served as the positive control for T-cell proliferation. Cells incubated in medium alone served as the negative control. After 72 h of incubation, cells were pulsed for 18 h with [^{3}H]thymidine (1 μCi/well; Amersham Corp., Arlington Heights, Ill.). Cells were harvested onto glass fiber filters, and the counts per minute due to [^{3}H]thymidine incorporation into DNA were measured by scintillation counting.

TABLE 1. Virulence of *E. faecalis* variants administered into the left ventricles of rabbits[a]

Test organism (phenotype)	No. of animals with clinical signs/no. evaluated	No. dead/no. evaluated (*P*)[b]
INY3000(pWM401) (AS^- EBS^-)	0/6	0/6
OG1SSp(pWM401) (AS^- EBS^+)	6/6[c]	1/6 (NS[d])
INY3000(pINY 1801) (AS^+ EBS^-)	3/3[c]	0/3 (NS)
OG1SSp (pINY1801) (AS^+ EBS^+)	9/9	9/9[e] (0.0002)

[a] American Dutch belted rabbits (1 to 2 kg) were injected intracardially with 1 ml (approximately 10^8 CFU) of test organism in their left ventricles. Animals were monitored for 2 days for signs of illness and death.

[b] *P* values were determined by Fisher's exact probability test in comparison to the number of deaths in the AS^- EBS^- group.

[c] Animals developed significant fevers (>40°C), diarrhea, and clear amber urine and had mottled faces. Upon examination of tissues, all animals had marked pyogenic infection of the pericardial sac. Other tissues appeared to be grossly normal.

[d] NS, not significant.

[e] Animals developed significant fevers (>40°C), diarrhea, and clear amber urine and had mottled faces. Upon autopsy, all of these animals showed extensive destruction of the pericardial sac and lungs. There was no sign of inflammation in any rabbit. One animal underwent premature euthanasia, as required by the University Animal Care Committee, because it was unresponsive to external stimuli and unable to right itself.

Cytokine assays. Cell-free culture fluids from human lymphocytes that had been stimulated for 4 days with enterococcal fractions, TSST-1, or negative controls were evaluated for gamma interferon and TNF-β as a measure of significant $CD4^+$ T-cell stimulation. The same culture fluids were evaluated for TNF-α as a functional measure of macrophage activation. For all assays, lymphocyte culture fluids from four comparably treated wells were pooled. All assays were performed with enzyme-linked immunosorbent assay (ELISA) kits purchased from R & D Systems, Inc., Plymouth, Minn. Assays were performed as suggested by the manufacturer.

Enterococcal culture supernatants and cell extracts. *E. faecalis* AS^+ EBS^+ and AS^- EBS^- organisms were cultured in 100 ml of dialyzable beef heart medium overnight at 37°C. Stationary-phase cultures were centrifuged at 500 × *g* for 15 min, and supernatants were filtered through 0.45-μm-pore-size filters (Costar Scientific, Cambridge, Mass.). Supernatants were treated with 400 ml of absolute ethanol at 4°C to precipitate proteins.

Cell pellets from the initial centrifugation described above were washed one time with 10 ml of 0.15 M NaCl and repelleted by centrifugation (500 × *g*, 15 min). Cells were suspended in 25 ml of TE buffer (10 mM Tris, 1 mM EDTA [pH 7.4]) and incubated with mutanolysin (50 μl of a 1-mg/ml solution) for 50 min at 37°C. Finally, cell extracts were treated with 100 ml of absolute ethanol at 4°C.

After 2 days, the precipitates from both sets of culture supernatants and mutanolysin-treated cells were collected by centrifugation (500 × *g*, 15 min), dried to remove residual ethanol, and finally resuspended in 1 ml of pyrogen-free distilled water.

Statistics. Fisher's exact probability test was used to evaluate significant differences in survival rates among groups. Vegetations were considered 1+ when they were pinpoint in size and only one or two were present. In contrast, 3+ vegetations were large (up to 5 mm in diameter), often obstructing the ascending aorta, and typically numerous. Intermediate vegetations were scored 2+. Vegetation quality was scored by at least two individuals in a blinded fashion.

RESULTS

First, we assessed the virulence of various strains of *E. faecalis* by injecting approximately 10^8 CFU into the left ventricles of American Dutch belted rabbits and observing them for 2 days for signs of illness and death (Table 1). AS^- EBS^- strain INY3000(pWM401) caused clinical signs in zero of six animals, and none of the animals died. Upon autopsy, no signs of infections were seen. AS^- EBS^+ strain OG1SSp(pWM401) caused clinical signs in six of six rabbits, but only one of six died. Autopsy evaluations of these animals revealed highly significant pericardial inflammation, as determined by direct microscopic examination of fluid for polymorphonuclear leukocytes (PMNs). AS^+ EBS^- strain INY3000(pINY1801)

TABLE 2. Rabbit splenocyte proliferation induced by cell-associated extracts of *E. faecalis* AS^+ EBS^+ and AS^- EBS^- organisms

Fraction tested	Mean incorporation of [^{3}H]thymidine ± SE (cpm)
None	10,200 ± 700
TSST-1 (1 μg)	249,200 ± 31,000
AS^+ EBS^+	
Undiluted	34,500 ± 2,900
1:10 dilution	62,400 ± 13,000
1:100 dilution	123,700 ± 14,000
1:1,000 dilution	194,900 ± 18,000
10^{-4} dilution	32,900 ± 3,700
AS^- EBS^-	
Undiluted	16,000 ± 900
1:10 dilution	41,300 ± 3,000
1:100 dilution	40,600 ± 3,100
1:1,000 dilution	49,200 ± 4,200
10^{-4} dilution	36,000 ± 14,000

TABLE 3. Human peripheral blood mononuclear cell mitogenicities of cell-associated extracts of *E. faecalis* AS^+ EBS^+ and AS^- EBS^- organisms

Fraction tested	Mean incorporation of [^{3}H]thymidine ± SE (cpm)	
	Expt 1	Expt 2
None	2,980 ± 260	1,550 ± 180
TSST-1 (1 μg)	35,500 ± 6,100	49,240 ± 1,330
AS^+ EBS^+		
Undiluted	8,300 ± 470	5,200 ± 430
1:10 dilution	30,900 ± 5,970	28,700 ± 1,520
1:100 dilution	2,200 ± 260	30,460 ± 2,050
1:1,000 dilution	2,600 ± 190	15,020 ± 1,430
AS^- EBS^-		
Undiluted	3,510 ± 160	ND[a]
1:10 dilution	3,280 ± 320	ND
1:100 dilution	3,230 ± 40	ND

[a] ND, not done.

caused clinical signs in three of three rabbits, and none died. As with AS^- EBS^+ organisms, rabbits which had received AS^+ EBS^- organisms showed significant pericardial inflammation. AS^+ EBS^+ strain OG1SSp(pINY1801) caused clinical signs in nine of nine animals, and all of the animals died. Necropsy analyses of these animals indicated no evidence of pericardial inflammation. Rather, grossly observable destruction of both myocardial and lung tissues was evident. These results indicate that in this model, strains which lack AS and EBS are relatively avirulent compared to strains that possess both of these traits and strains which possess either AS or EBS are intermediate in virulence by comparison. Furthermore, this experiment suggested that the AS^+ EBS^+ strain produced factors that prevented pericardial inflammation, either through destruction of inflammatory cells or by preventing PMN influx into the site of infection.

Since the *E. faecalis* strains used in this study lack hemolysin and do not make known factors that are toxic to PMNs, it was hypothesized that AS^+ EBS^+ organisms made one or more factors that induced lethality and prevented PMN influx into the heart. Superantigens made by both *Staphylococcus aureus* and group A streptococci have previously been shown to cause high-level release of both TNF-α and -β from macrophages and T cells, respectively, both of which cause capillary leak and down regulate chemotactic receptors on PMNs (16, 17, 24). The latter effect of superantigens has previously been shown in in vitro systems to prevent chemotaxis of PMNs and has been proposed in part to explain the lack of inflammation seen at sites of infection during both staphylococcal and streptococcal toxic shock syndromes (TSS) (3, 29). Thus, it was possible that AS^+ EBS^+ *E. faecalis* organisms made superantigen-like molecules that had a similar lethal and antichemotactic effect.

In order to test for superantigen-like molecules, culture supernatants and cell extracts from both AS^+ EBS^+ and AS^- EBS^- organisms were evaluated for the ability to stimulate rabbit and human lymphocytes. Cell-free culture fluids from both organisms failed to stimulate either rabbit or human lymphocytes (data not shown). In contrast, TSST-1 (used as a positive control) caused highly significant proliferation. Cell extracts of AS^+ EBS^+ organisms also caused significant lymphocyte proliferation in both species (Tables 2 and 3). As indicated in Table 2, AS^+ EBS^+ organisms induced the highest proliferation of rabbit lymphocytes at the 1:1,000 dilution of cell extract, with activity comparable to that of the superantigen TSST-1 (used as a positive control). In contrast, cell extracts from AS^- EBS^- organisms caused significantly less rabbit lymphocyte proliferation at all of the dilutions tested.

AS^+ EBS^+ organisms also caused significant human peripheral blood lymphocyte proliferation, with the peak proliferation occurring with the 1:10 diluted extract. The response seen at that dilution was again comparable to the response seen with TSST-1. Extracts from AS^- EBS^- organisms failed to stimulate proliferation of human lymphocytes. In both rabbit and human test systems, high doses of extract caused inhibition of lymphocyte proliferation.

Superantigenic stimulation of lymphocytes results in massive releases of cytokines, such as TNF-α from macrophages and TNF-β and gamma interferon from $CD4^+$ T cells (16, 17, 24). Release of these cytokines can also be used as an indicator of whether the lymphocyte proliferation that occurred was the result of functional activation of T cells and macrophages. The 1:10 diluted cell extract from AS^+ EBS^+ organisms, which was the dilution which induced maximal human lymphocyte proliferation, caused highly significant release of each of these three human cytokines (Table 4). Thus, cell extracts of AS^+ EBS^+

TABLE 4. Ability of cell-associated extract of *E. faecalis* AS^+ EBS^+ organisms to induce cytokine release from human peripheral blood mononuclear cells, as measured by ELISA

Test material	TNF-α (pg/ml)	TNF-β (pg/ml)	Gamma interferon (pg/ml)
Negative control	25	16	8
TSST-1 (1 μg)	>1,000[a]	800	>1,000[a]
AS^+ EBS^+ (1:10 dilution)	5,000[b]	625	5,500[b]

[a] TSST-1 stimulation of human peripheral blood mononuclear cells was used as the positive control. Undiluted human cell culture fluids were tested for these cytokines, and the amounts exceeded the upper detection limit (1,000 pg/ml) of the ELISA.

[b] Lymphocyte culture fluids were diluted 1:10 prior to assays of these cytokines for more precise measurements of cytokine levels; only undiluted positive control culture fluids were tested. Thus, data for these cytokines were multiplied by the dilution factor of 10.

TABLE 5. Abilities of *E. faecalis* variants to cause endocarditis in an experimental rabbit model[a]

Test organism (phenotype)	No. of animals with vegetations/ no. evaluated (*P*)[b]	Quality of vegetations	Other tissue(s) affected
INY3000(pWM401) (AS^- EBS^-)	0/4[c]	None	None
OG1SSp)pWM401) (AS^- EBS^+)	4/4 (0.014)	Very small (1+)	Spleen enlargement
INY3000(pINY1801) (AS^+ EBS^-)	4/4 (0.014)	Very small (1+)	Spleen enlargement
OG1SSp(pINY1801) (AS^+ EBS^+)	4/4[c] (0.014)	Large (3+)	Spleen enlargement, lung congestion

[a] New Zealand White rabbits (4 to 6 kg) underwent transaortic catheterization for 2 h and were injected intravenously through their marginal ear veins with approximately 2×10^9 CFU. Animals were sacrificed on day 3 and examined for heart vegetations and signs of infection in other tissues.
[b] *P* values were determined by Fisher's exact probability test in comparison to the number of animals with vegetations in the AS^- EBS^- group.
[c] Two additional animals were injected with 5×10^9 CFU. Neither of the two animals given INY3000(pWM401) succumbed or showed vegetations in the heart. Both animals given OG1SSp(pINY1801) succumbed overnight and showed extensive lung necrosis.

organisms induced T-cell proliferation, with consequent release of TNF-β and gamma interferon and activation of macrophages to release TNF-α.

Next, we investigated the abilities of the same strains to cause cardiac vegetations in a rabbit catheterization model. New Zealand White rabbits were anesthetized, and each had a catheter placed across the aortic valve via the left carotid artery. The catheter was left in place for approximately 2 h and subsequently removed prior to intravenous injection of 2×10^9 CFU of test organisms. The animals were sacrificed on day 3 and examined for cardiac vegetations or other organ damage (Table 5). The AS^- EBS^- strain produced vegetations in zero of four animals and affected no other organ. Two additional animals given 5×10^9 CFU of the AS^- EBS^- strain had identical negative results. The AS^- EBS^+ and AS^+ EBS^- strains produced very small cardiac vegetations and enlarged spleens in all of the animals tested (four each). The AS^+ EBS^+ strain produced large vegetations, and five of the five animals tested had enlarged spleens and lung congestion. Two additional animals given 5×10^9 or 2×10^{10} CFU of the AS^+ EBS^+ strain succumbed overnight and showed extensive lung necrosis. As in the previous experiment, these results show that in this model, strains which possess both AS and EBS are more virulent and have an enhanced ability to cause cardiac vegetations compared to those of strains which lack AS and EBS. Strains with either AS or EBS are virulent and can produce vegetations but to a lesser degree than AS^+ EBS^+ strains can.

We also investigated the ability of some of these organisms to cause endocarditis in the same model, modified by leaving the catheter in place for the 3-day observation period. Rabbits were prepared in a similar fashion, except that the catheter was placed through the aortic valve and not removed prior to injection of 2 ml of test organism at approximately 2×10^9 CFU. The AS^- EBS^- strain caused small (1+) vegetations in three of four animals. The other animal, whose myocardium had been completely penetrated by the catheter, had very large vegetations. The effects on other organs were minimal to none in all of these animals. The AS^+ EBS^+ strain caused very large (3+) vegetations and lung congestion in four of four animals. These experiments, taken together with the previous results, indicate that both AS and EBS are important in the ability of this organism to cause cardiac vegetations; however, leaving transaortic catheters in place during infection may artificially enhance the capacities of test strains to cause cardiac vegetations in this model.

DISCUSSION

Here, two models for the study of *E. faecalis* endocarditis were established. Experiments demonstrated that both AS and EBS were important in establishing cardiac infections.

In the first model, organisms were injected directly into the left ventricle of the heart. Although this is not the standard model for the induction of endocarditis in rabbits, the experiment clearly showed differences in virulence among enterococcal strains. The most interesting aspect was the observation that rabbits injected with AS^+ EBS^+ organisms succumbed, whereas rabbits given AS^- EBS^- organisms showed no signs of infection. It is clear from these studies that both enterococcal cell surface receptors for mating pair formation contribute to virulence. Animals that received AS^+ EBS^+ organisms developed a TSS-like illness and succumbed with major destruction of heart and lung tissues but without signs of pericardial inflammation. In TSS, a similar lack of inflammation has previously been proposed to result from massive TNF release as a result of superantigen activity, with consequent down regulation of PMN chemotactic receptors and failure of PMNs to migrate into the infected area (17, 18, 24). AS^+ EBS^+ organisms may have made a superantigen that caused some of the biological effects seen. This is consistent with the highly significant rabbit and human lymphocyte proliferation induced by AS^+ EBS^+ organisms, which was comparable to that seen with TSST-1. Furthermore, lymphocyte proliferation involved T cells since high levels of TNF-β and gamma interferon were released, a result consistent with that expected for T-cell superantigens (16, 17). This is also consistent with three cultures of enterococci submitted to us from blood samples of patients with TSS for which enterococci were thought to be the cause. It is likely that the T-cell stimulatory factors present in AS^+ EBS^+ organisms but not in AS^- EBS^- organisms were either aggregation or binding substances or more likely both, since these organisms have the same genetic background but differ in those two factors. A previous study of enterococcal LTA, which is likely to be analogous to enterococcal EBS, showed that LTA causes significant TNF-α release (7). It is unlikely that the effects observed were due to the toxic effects of peptidoglycan since both organisms have this molecule. It is also possible that the observed effects of the AS and EBS phenotypes on virulence are due to an indirect effect of these components on the expression or activities of other surface factors. However, the data presented here, along with previous results (9, 21, 25), clearly demonstrate major effects of AS and EBS on enterococcal virulence.

In the same intraventricular injection model, both AS^+ EBS^- and AS^- EBS^+ organisms showed intermediate virulence, indicating the importance of both factors in complete activity. Finally, organisms that lacked both factors appeared to be cleared very rapidly from rabbits so that no evidence of infection was observable.

In the second model, animals were injected with the same enterococcal strains after transaortic catheterization. This model has previously been used by other investigators to study bacterial endocarditis (4, 9) and to demonstrate the impor-

tance of AS in virulence (9). For example, in a study by Chow et al. (9), it was demonstrated that both AS and hemolysin contributed to increased mortality in experimental endocarditis and that AS was associated with increased vegetation weight. In our studies, four major observations were made. First, inoculum size was important, particularly in the case of AS^+ EBS^+ organisms. For example, an administration of 5×10^9 or greater CFU of the AS^+ EBS^+ strains resulted in death, whereas doses of 2×10^9 CFU or below did not. This high-dose effect is likely comparable to that seen when organisms were injected intraventricularly at the 10^8 dose since similar gross pathological changes were seen. In contrast, AS^- EBS^- organisms did not cause lethality at any dose, similar to what was seen in our previous experiments. Second, catheter size was important. An administration of AS^+ EBS^+ organisms at doses of 2×10^9 CFU intravenously to rabbits without catheters or with small-internal-diameter catheters failed to result in signs of infection. Third, continuous exposure of rabbits to catheters resulted in greater sizes and numbers of vegetations in the heart and rendered AS^- EBS^- organisms capable of causing endocardial disease. Lastly, AS^+ EBS^+ organisms caused more significant cardiac infections, typified by large and numerous vegetations, than did either organisms having only one of the two factors or organisms lacking both. Organisms having either AS or EBS were intermediate in the ability to cause cardiac vegetations. Thus, we confirmed the findings of Chow et al. (9) concerning the role of AS in virulence of *E. faecalis* but also extended those findings to include the combination of AS and EBS. It is also important that Chow et al. (9) demonstrated the importance of hemolysin in disease. The organisms used in our studies lacked hemolysin production.

The heart valve vegetations characteristic of both clinical endocarditis in humans and experimental endocarditis in animals result from a complex series of interactions among damaged cardiac endothelium, bacteria, platelets, and serum factors (1). Our results also suggest that a cell surface-associated bacterial superantigen or another type of mitogenic factor contributed to the lethality observed after direct injection of AS^+ EBS^+ organisms into the heart (Table 1) and in some catheterized animals which experienced severe endocarditis and death after infection with the same strain (Table 5). Based on the data obtained thus far, it is likely that several potential virulence properties, including adherence, resistance to killing by phagocytes, and production of toxins, are related to the AS and EBS phenotypes of *E. faecalis*. Further biochemical and genetic analyses of these surface components are required to define the molecular basis for the correlation of the AS^+ EBS^+ phenotype with virulence in this system. Finally, it should be noted that the AS^+ strains used here are recombinant strains which express Asc10 constitutively. Our recent evidence (unpublished data) suggests that a host factor present in both rabbit blood and human blood can increase the expression of Asc10 in cells carrying wild-type pCF10. This is consistent with a previous report of in vivo induction of AS expression (6).

Thus, in two models of enterococcal infections, organisms with both AS and EBS exhibited the greatest ability to cause disease, with AS^+ EBS^- and AS^- EBS^+ organisms demonstrating intermediate virulence and AS^- EBS^- organisms lacking the ability to cause disease (except in instances where catheters remained in rabbits). It is, however, important to remember that available data indicate that some clinical isolates of enterococci from patients with endocarditis apparently lack AS. Therefore, other factors must also play important roles in the clinical setting. One possible example of such a factor is the chromosomally encoded EfaA antigen (22), which is similar in sequence to the FimA adhesion protein, previously shown to contribute to virulence in *Streptococcus parasanguis* experimental endocarditis (8, 33).

Future studies are directed toward evaluating AS and EBS, both alone and in combination, for superantigen activity. In addition, we are attempting direct purification of the putative superantigen from the AS^+ EBS^+ strain. Final clarification of whether the purified substance is a superantigen will require the determination of whether the factor has the following defining superantigen properties: (i) activation of T cells independent of antigen specificity, (ii) ability to activate T cells dependent on the variable region of the β chain of the T-cell receptor, and (iii) requirement for presentation to T cells via antigen-presenting cells without restriction.

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