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Binding of Viridans Group Streptococci to Human Platelets: a Quantitative Analysis†

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The binding of viridans group streptococci with human platelets was analyzed by two-color flow cytometry. Binding was detected within 15 s of mixing bacteria and platelets. At ratios of bacteria to platelets of 1:1, 10:1, 100:1, and 1,000:1, the percentages of bound streptococci (mean ± standard deviation) were 93.2% ± 5.4%, 80.0% ± 8.6%, 39.8% ± 11.1%, and 12.5% ± 2.0%, respectively. Binding of labeled bacteria was reversed by adding a 500-fold excess of unlabeled streptococci. These results demonstrate that streptococcus-platelet binding is rapid, reversible, and saturable, which suggests a specific receptor-ligand interaction.

The binding of platelets and bacteria is a postulated central event in the pathogenesis of endocarditis. This process may facilitate colonization of the valve surface by enhancing the adherence of blood-borne microorganisms (11, 16). In addition, platelet-bacterium binding may be an important mechanism for production of the macroscopic endocardial vegetation (4, 6, 17). Although histologic and turbidometric studies have provided inferential evidence for the binding of microorganisms to human platelets, it has been difficult to measure binding directly. To determine more definitively whether bacterium-platelet binding occurs and to assess this interaction quantitatively, we have used flow cytometry to analyze the binding of viridans group streptococci with human platelets.

Four strains of viridans group streptococci were assessed for their binding to platelets. D1 and M99 are endocarditis-producing strains of *Streptococcus salivarius* and *Streptococcus sanguis* I, respectively. Strains Challis and C261 are two laboratory isolates of *S. sanguis* I. Overnight cultures of each strain were washed three times and labeled at 4°C with 100 μg of Hoechst 33342 per ml (5, 20). After 3 h, the bacteria were washed, suspended in Tyrode solution (pH 7.4), and sonicated (19).

Platelets in plasma were isolated from human blood by centrifugation (19). For most experiments, platelets were labeled at 20°C with 2.5 μg of fluorescein isothiocyanate (FITC)-conjugated monoclonal antibody P2 per ml (13). After 2 h, the platelets were washed twice and suspended in Tyrode solution. In some studies, platelets were labeled directly with 25 μg of 5-(4,6-dichlorotriazinyl)aminofluorescein (DTAF) per ml in phosphate-buffered saline, pH 7.4 (1).

A modified FACS IV cytofluorograph and cell sorter (Becton Dickinson, Mountain View, Calif.) was used to detect the binding of streptococci to platelets. Fluorescein was excited at 501 nm with an argon laser (Spectra-Physics, Inc., Mountain View, Calif.) operating at 300 mW, and the emitted light was collected through a 525 ± 25-nm band pass filter. Hoechst 33342 was excited at 363 nm by a second argon laser, and the emitted light was collected through a <460-nm short-pass filter. Suspensions of unlabeled and labeled bacteria alone and platelets alone were then analyzed, and two-dimensional fluorescence profiles were produced according to the schema shown in Fig. 1, with a DEC PDP11/73 computer (Digital, Merrimack, N.H.) and specially designed software (Becton Dickinson).

Binding studies were performed at 20°C by mixing bacteria (10^6 to 10^8) and platelets in 1 ml of Tyrode solution and were done over a range of bacterium/platelet ratios (1:20 to 1,000:1). At preselected times after mixing, the samples were analyzed by flow cytometry. The percentage of bacteria bound to platelets was calculated by dividing the number of dually labeled particles (representing bacteria bound to platelets) by the total number of particles labeled with Hoechst 33342 (i.e., bound and unbound bacteria) and multiplying by 100. Data are expressed as means ± standard deviations and represent multiple experiments performed on different days. Unless indicated otherwise, differences in the mean number of bacteria bound to platelets were compared by Student's *t* test.

Flow cytometry of bacteria (Fig. 2A) or platelets (Fig. 2B) alone revealed that each cell type was well labeled and appeared as a single population. No overlap was seen in the emission spectra of labeled platelets and bacteria. To assess binding, strain D1 (4×10^6 organisms) was mixed for 30 min with an equal number of platelets in 1 ml of Tyrode buffer. A new population of dually labeled particles (Fig. 2C) was observed, indicating that binding had occurred. At this ratio of bacteria to platelets (1:1), 90.7% ± 6.5% of the streptococci were bound to platelets (seven experiments, with platelets from a different donor on each occasion). Binding of bacteria and platelets was detectable within 15 s after mixing, and the percentage of bacteria bound after 30 s (93.2% ± 5.4%) was comparable to that observed at later times (1 to 15 min; $n = 5$).

To determine whether binding was reversible, strain D1 (4×10^6 cells) was mixed with an equal number of platelets. After 30 min, 2×10^9 unlabeled bacteria per ml (i.e., a 500-fold excess) were added to the suspension. Five minutes

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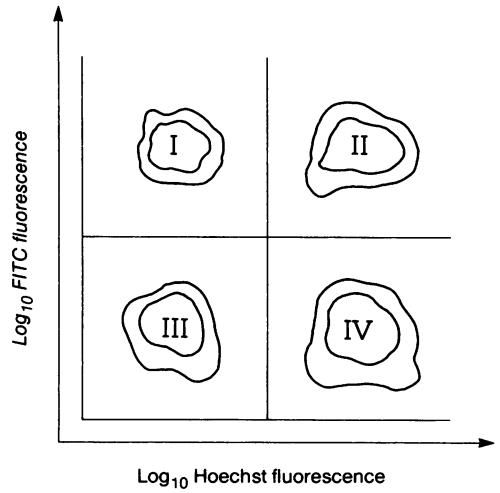

FIG. 1. Schematic representation of flow cytometric profile for two-color fluorescence. Quadrants I through IV outline the four possible cell phenotypes. Quadrant I, Particles labeled with FITC alone (unbound platelets); quadrant II, particles labeled with FITC and Hoechst 33342 (bacterium-platelet complexes); quadrant III, unlabeled particles; quadrant IV, particles labeled with Hoechst alone (unbound bacteria).

later, the sample was diluted 1,000-fold and analyzed immediately by flow cytometry. Addition of unlabeled bacteria significantly reduced the percentage of previously bound, labeled bacteria from 94.6% ± 5.0% to 35.4% ± 6.4% ($n = 5$, $P < 0.01$), indicating that binding was reversible.

The binding of bacteria and platelets was also saturable. Platelets (4×10^6) were incubated with 4×10^8 unlabeled streptococci for 30 min. Labeled D1 (4×10^6 bacteria) was then added, and binding was assessed. In control studies with platelets preincubated in Tyrode solution alone, 91.0% ± 6.1% of bacteria were bound. However, preincubation of platelets from the same donors with unlabeled bacteria reduced subsequent binding by labeled organisms to 36.6% ± 15.8% ($P < 0.01$, $n = 4$). Binding was also examined at various ratios of bacteria to platelets (Fig. 2D and 3). At streptococcus/platelet ratios of 1:20 to 5:1, the percentage of bacteria bound to platelets remained above 85%. However, as the ratio was increased, the percentage of bacteria bound declined. At ratios of 10:1, 100:1, and 1,000:1, the percentages of bacteria bound to platelets decreased to 80.0% ± 8.6%, 39.8% ± 11.1%, and 12.5% ± 2.0%, respectively, further indicating that saturation had occurred.

To confirm that flow cytometry measured binding accurately, we performed quantitative cultures on the populations identified as bound and unbound bacteria. Strain D1 and platelets were mixed for 1 min at a ratio of 5:1. Analysis by flow cytometry indicated that 97.0% of the streptococci were bound to platelets. The unbound bacteria and bacterium-platelet complexes were collected into separate sterile tubes with the cell sorter. After 10 s of sonication (to disperse possible clumps), samples from each tube were plated onto blood agar and incubated at 37°C for 24 h. As measured by quantitative cultures, 98.8% of the total bacteria had bound to platelets, a value nearly identical to that obtained by flow cytometry.

Since certain streptococci can bind the Fc region of immunoglobulin G (IgG) (14, 21), we were concerned that the monoclonal antibody used to label the platelets could serve as a ligand for the bacteria. To assure that the observed binding of platelets and bacteria was not an artifact of our labeling methods, we evaluated the binding of strain D1 to platelets that had been labeled directly with DTAF. Platelets and bacteria were mixed at a ratio of 1:1 for 10 min and then analyzed by flow cytometry, as described above. As a control, binding studies were also performed with antibody-labeled platelets from the same donors. When tested with DTAF-labeled platelets, 85.8% ± 6.7% of bacteria were bound; a similar percentage (91.8% ± 3.2%) of bacteria was bound to platelets labeled with monoclonal antibody P2 ($n = 3$). Thus, binding was independent of the method employed for labeling platelets and was not mediated by the FITC-conjugated monoclonal antibody.

Our previous studies have shown that platelet aggregation by viridans group streptococci requires IgG specific for the bacteria and that IgG binding to platelet Fc receptors contributes to activation (18). To determine whether Fc receptors participated in streptococcal binding, platelets were incubated for 30 min in 50 ng of monoclonal antibody IV.3 per ml, which binds the 40,000-M_r platelet receptor for the Fc region of human IgG (15). The platelets were then mixed with strain D1 at bacterium/platelet ratios of 1:1. Preincubation of platelets with monoclonal antibody IV.3 produced no significant change in the binding of strain D1 (84.1% ± 4.6% bound, $n = 4$). However, in studies with platelets from the same donors, 50 ng of this antibody per ml completely blocked platelet aggregation by strain D1. (Aggregometry was performed as described previously [2, 19].)

To better characterize the streptococcal component that mediates binding, we evaluated suspensions of strain D1 that had undergone 1 h of digestion (3, 12) with the following enzymes (all obtained from Sigma Chemical Co., St. Louis, Mo.): proteinase K, 2 mg/ml (type XI); trypsin, 4 mg/ml (type III); chymotrypsin, 1 mg/ml (type VII); and neuraminidase, 4 mg/ml (type II). Binding studies were performed at bacterium-to-platelet ratios of 1:1 and 10:1. After being mixed for 1, 10, or 15 min, the samples were analyzed by flow cytometry. Treatment of strain D1 with these enzymes produced no significant change in the percentage of bacteria bound to platelets ($n = 4$). Even when proteinase K digestion was extended to 4 h, binding remained unchanged. Similar results were observed with other strains of viridans group streptococci. In studies with untreated bacteria, 90.1% ± 5.2% of strain M99, 90.6% ± 4.5% of the Challis strain, and 88.9% ± 3.0% of strain C261 were bound to platelets by 1 min. Digestion of these organisms with proteinase K for 1 h failed to alter their binding to platelets.

The effect of these enzymes on platelet aggregation by streptococci was also evaluated. Addition of either untreated strain D1 or M99 to platelets resulted in an 8- to 13-min lag phase, followed by rapid and maximal platelet aggregation (19). Digestion of these strains for 1 h with proteases or neuraminidase had no effect on their ability to aggregate platelets as measured by duration of the lag phase, rate of aggregation, or absolute change in light transmission ($n = 3$ experiments).

We then examined whether treating platelets with chymotrypsin or neuraminidase altered binding to strain D1. Digestion of platelets with chymotrypsin for 1 h had no effect on binding. However, platelets treated with neuraminidase were bound slightly less effectively by D1. When mixed at a ratio of 10:1 (bacteria to platelets), 85.0% ± 12.2% of bacteria were bound to untreated platelets. However, only 70.4% ± 18.6% of bacteria incubated with neuraminidase-treated platelets were bound ($P < 0.009$, paired t test, $n = 5$).

Binding of streptococci with platelets was characterized

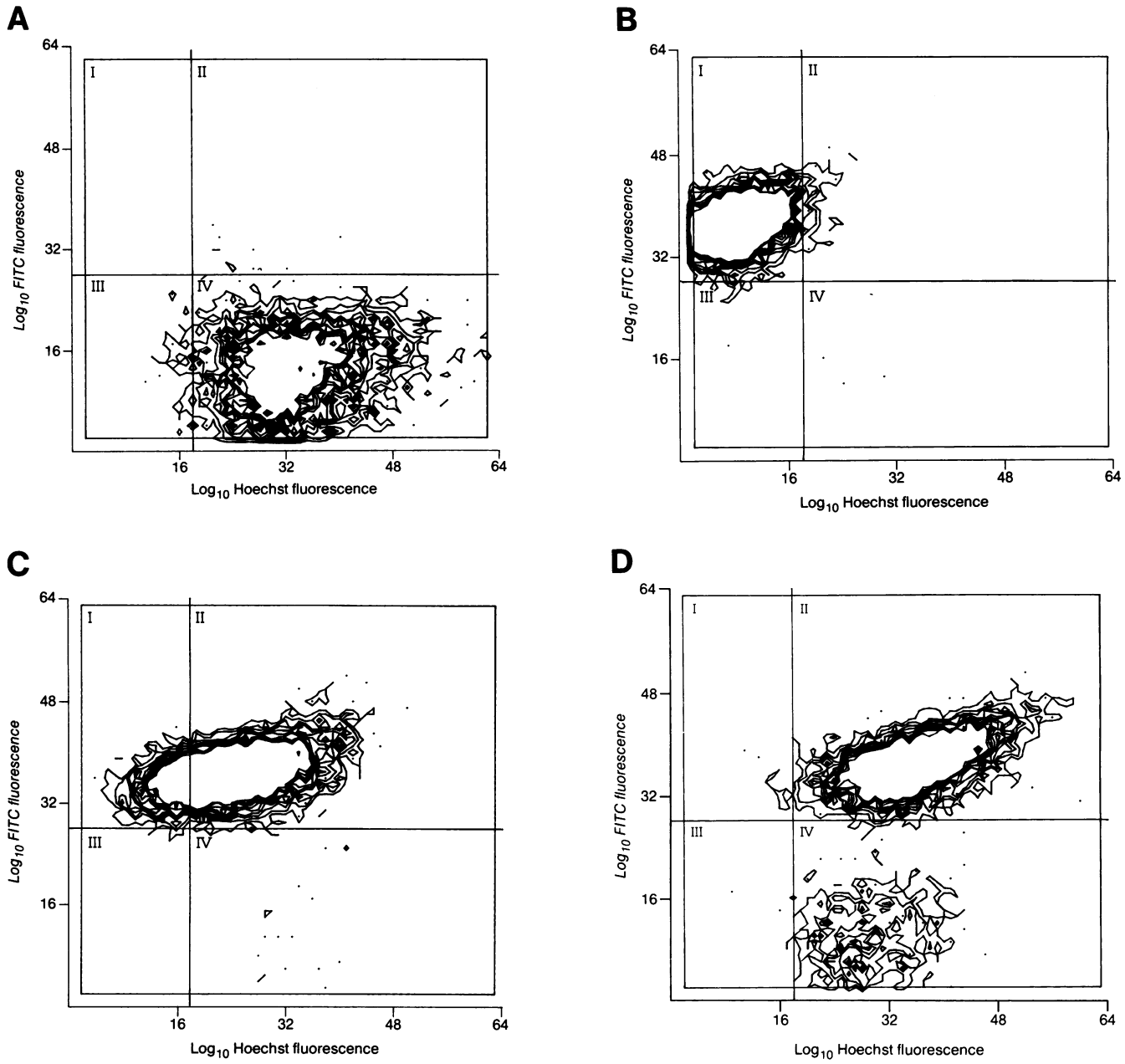

FIG. 2. Two-color analysis of streptococcus-platelet binding. (A) Strain D1 labeled with Hoechst 33342. The bacteria appear in quadrant IV as a single population of well-labeled particles. (B) Platelets labeled with FITC-conjugated monoclonal antibody P2. The platelets are seen as a single population of FITC-labeled cells. Over 95% of the platelets are contained in quadrant I. (C) Strain D1 plus platelets. Bacteria and platelets were mixed at a ratio of 1:1 and subjected 30 min later to flow cytometry. A new population of dually labeled particles has emerged in quadrant II, indicating that binding has occurred. (D) Strain D1 plus platelets, mixed at a bacterium/platelet ratio of 10:1. Over 99% of the platelets have bound bacteria. Additional unbound bacteria are now observed in quadrant IV, indicating that saturation of binding is occurring.

further by transmission electron microscopy. Strain D1 and washed platelets were mixed for 30 min at a ratio of 1:1, fixed with 1.5% glutaraldehyde in 0.1 M sodium cacodylate–1% sucrose (pH 7.4), and processed for morphologic study (10). By electron microscopy, bacteria were observed in complexes with platelets (Fig. 4). For some organisms, the bacterial cell wall could be seen in direct contact with the platelet membrane.

Our data indicate that viridans group streptococci can adhere to platelets directly and that binding is rapid, reversible, and saturable. These findings suggest a ligand-receptor interaction. However, if a single class of streptococcal surface components mediates binding to platelets, the chemical nature of the ligand is unclear. Digestion of bacteria with proteinase K, trypsin, or chymotrypsin had no effect on binding, indicating that this interaction is not mediated by a streptococcal protein. In contrast, Herzberg et al. found that even brief treatment of one strain of *Streptococcus sanguis* with proteases reduced its ability to agglutinate platelet ghosts, as measured by a turbidometric assay (8, 9). These

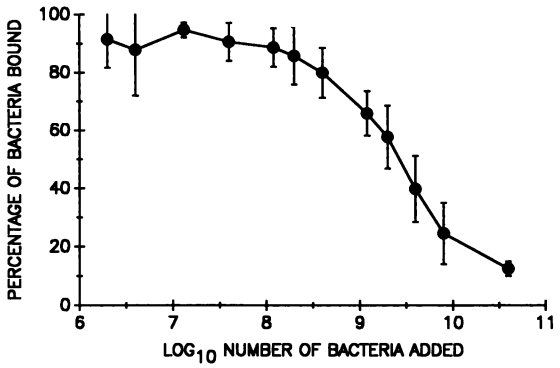

FIG. 3. Saturation of platelet binding by strain D1. Bacteria and platelets were mixed at various ratios, and the percentage of streptococci bound was calculated. At bacterium/platelet ratios of 1:20 to 1:1 ($\log_{10}$ number of bacteria added, 6.3 to 7.6), over 85% of bacteria were bound to platelets. At ratios beyond 5:1 ($\log_{10}$ = 8.3), the percentage of bacteria bound declined progressively, indicating that saturation was occurring.

conflicting results could be due to differences in the methods used to detect binding. Although platelet agglutination may reflect streptococcus-platelet binding, it is only an indirect measure of this process. Moreover, it is unclear whether the turbidometric assay described above detects interactions other than bacterium-platelet binding. Alternatively, the mechanisms by which streptococcal strains bind to platelets may vary, as has been shown for the adherence of *S. salivarius* strains to buccal epithelial cells (7, 22).

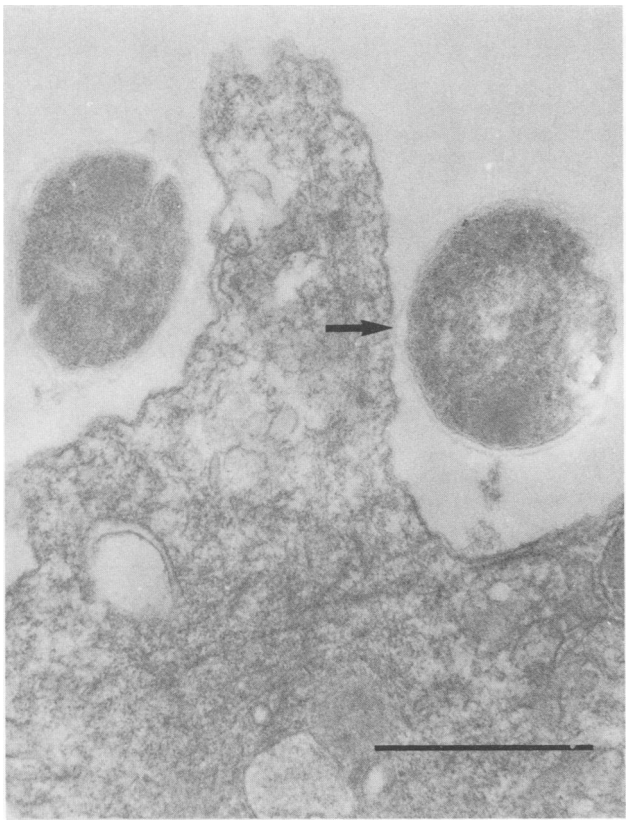

FIG. 4. Ultrastructural analysis of streptococcus-platelet binding. Strain D1 and platelets were mixed for 30 min at a ratio of 1:1 and then fixed. Bacterium-platelet complexes can be seen, and for some organisms, the cell wall appears in direct contact with the platelet membrane (arrow). Bar = 0.5 μm.

Our previous studies suggested that platelet aggregation by viridans group streptococci consisted of two sequential processes: binding of platelets and bacteria, and platelet activation, which required IgG specific for the organisms (18, 19). By means of flow cytometry, we have now demonstrated that viridans group streptococci can bind platelets directly. Further studies are needed to define the molecular basis of binding, to characterize its role in platelet aggregation by streptococci and to determine its significance in the pathogenesis of streptococcal endocarditis.

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