Direct Flow Cytometry of Anaerobic Bacteria in Human Feces

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We describe a flow cytometry method for analysis of noncultured anaerobic bacteria present in human fecal suspensions. Nonbacterial fecal compounds, bacterial fragments, and large aggregates could be discriminated from bacteria by staining with propidium iodide (PI) and setting a discriminator on PI fluorescence and by exclusion of events with large forward scatter. Since anaerobic bacteria, which account for over 99.9% of all fecal bacteria, die during sample preparation, a fixation step was not necessary.

A second aim of this study was to investigate the technical possibility of measurement of in vivo IgA coating of fecal anaerobic bacteria as well as their bacterial size. Fecal samples of 22 healthy human volunteers were analyzed. The fluorescence distribution of IgA-coated bacteria labeled with fluorescein isothiocyanate (FITC)-anti-Hu-IgA had overlap with noncoated bacteria. However, with match region subtraction, detection of low levels of specific FITC fluorescence on IgA-coated bacteria was achieved. The median bacterial two-dimensional surface area was 1.0 μm². To validate flow cytometry data, all samples were analyzed with an image analysis system as well. With this new method, a rapid evaluation of fecal flora with high sensitivity for specific FITC fluorescence is possible without culturing.

Key terms: Anaerobic bacteria, coproantibody, fecal flora, flow cytometry, IgA, image analysis, microflora, microorganisms

Flow cytometry offers a rapid method for the characterization of individual cells in mixed populations by physical and biochemical aspects. While major attention has been paid to measurement of eukaryotic cells, only some attempts have been made to analyze bacterial populations. In this field, the focus has been on bacterial pure cultures (2,6,16,18,24), though in some studies noncultured mixed bacterial populations such as aquatic bacteria were characterized (21,23). Flow cytometry may be a potential technique to analyze fecal flora as well. However, no reports on this subject have been published as yet.

There is growing interest in analysis and characterization of the anaerobic intestinal flora. Fecal anaerobic bacteria may play a role in the pathogenesis of diseases such as graft-vs.-host disease (3,26), inflammatory bowel disease (17,28), and bacterial cell wall-induced arthritis in rats (13). Furthermore, intestinal anaerobic bacteria may inhibit colonization of the intestinal tract by potentially pathogenic and pathogenic bacteria and thereby prevent infections (27).

The human colon harbors about 10¹¹ bacteria per gram contents. It is important to realize that over 99.9% of the colonic microflora consists of a stable ecosystem of possibly as many as 400 different species of anaerobic bacteria in a characteristic individual composition (7,8,15). This composition may be influenced by IgA that is secreted in large amounts into the intestinal lumen. Potentially pathogenic aerobic bacteria such as Enterobacteriaceae spp. (e.g., Escherichia coli) make up less than 0.1% of the colonic flora.

Fecal anaerobic bacteria are difficult to study. Culturing and identifying anaerobic bacteria by biochemical properties are very time consuming. Moreover, for immunological studies, culture of bacteria may change their antigenic expression and harbors the danger of a bias towards easily culturable bacteria (5,7,12,19,20). Therefore, we aimed to examine noncultured human

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fetal anaerobic bacteria. A few years ago, we developed a phase contrast-fluorescence microscope-based computer image analysis system that enables us to record morphology and immunofluorescence labeling of each fecal bacterium separately (1,10,29). This image analysis method, however, is quite laborious.

In the present study, we describe a new rapid method to characterize the anaerobic fecal flora by flow cytometry. Fecal samples from 22 healthy human volunteers have been examined, and forward scatter distributions are presented. Furthermore, we have determined the technical possibility of measurement of in vivo IgA coating of fecal anaerobic bacteria. To validate flow cytometry data, all samples were analyzed with a computer image analysis system as well.

**MATERIALS AND METHODS**

**Volunteers and Sampling**

Twenty-two healthy volunteers, 13 males and nine females, aged 21–61 years (median 32 years), provided a fecal sample. Exclusion criteria were: immunocompromised conditions (corticosteroids, diabetes, etc.), antibiotic use less than 2 weeks before sampling, diarrhea, and pregnancy. Each fecal sample was divided into 12 portions of 0.5 g, frozen within 3 h after defecation and stored until use at −20°C.

**Pure Cultures**

Pure cultures of human feces-derived *Bacteroides fragilis*, *Fusobacterium* spp., and *Clostridium difficile* were grown under anaerobic circumstances in chopped meat carbohydrate. Pure cultures of human-derived *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella* spp., *Pseudomonas* spp. were incubated in brain heart infusion growth (BHI; Oxoid, Hampshire, England). All strains were stored at −20°C until use.

**Reagents**

Affinity-purified fluorescein isothiocyanate (FITC)-labeled goat F(ab')₂ antihuman IgA (F/P ratio 2.0) from Kallestad (Austin, TX), FITC-labeled goat F(ab')₂ antimouse IgM (FIP ratio 3.2) from Protos immunoreheim, Germany), and propidium iodide (PI) from Sigma.

**Flow Cytometry**

**Instrumentation.** Flow cytometry was performed with an EPICS-ELITE (Coulter Electronics, Hialeah, FL). Sample excitation was done with an argon laser operating at 15 mW and 488 nm. Filter settings were 525 BP for FITC and 550 LP and 630 BP for measurement of PI. Acquisition and data analysis were done with standard ELITE software, using the Immuno-4 program to determine the percentage of stained events.

**Calibrations and discriminator.** The flow cytometer was calibrated with Fluoresbrite plain microspheres (Polysciences, Inc., Warrington, PA), 0.72 μm in diameter, on forward scatter (FSC), side scatter (SSC), and FITC fluorescence. Fluorescence quantitation was done with fluorescein quantitation kits (Quantum 24 and Quantum 25, from Flow Cytometry Standards Corp., Research Triangle Park, NC). To determine the level of background noise, we used plain microspheres (Polysciences, Inc.), 0.79 μm in diameter, which were assumed to have no fluorescence. For bacterial measurements, the discriminator was set on PI fluorescence as a specific probe for bacteria. The discriminator value was determined with a filtered bacteria-free (0.22 μm; Millipore, Molsheim, France) solution of PI/PBS (4 mg/liter) and set at a level with minimal background noise. For pure cultures of larger vital aerobic bacteria, a discriminator set on FSC was used, and its value was determined with filtered PI/PBS (4 mg/liter) at a level with minimal background noise.

**Actual analysis.** For each sample, a portion incubated with PBS (background fluorescence) and a portion incubated with FITC-labeled goat F(ab')₂ antihuman IgA were analyzed. Both measurements were performed with 10,000 events, at a flow rate of 1,000–1,500 events per second. Data were stored in list mode on disc. The fluorescence was recorded logarithmically, FSC linearly. The mean fluorescence (logarithmic scale) was the fluorescence value corresponding to the calculated mean channel number (linear scale) of all events and was therefore not a true mean. Percentages of stained bacteria were determined with Immuno-4 software (Coulter).

Sorting experiments were performed with gates on FSC >1,000 and on FSC <1,000 in combination with gates on SSC or PI. Furthermore, a sorting experiment with a FSC-FITC fluorescence gate was performed to isolate a strongly IgA-coated bacterial population. Sorted bacteria were collected on a slide and further evaluated via microscopy and computer image analysis.

**Isolation and preparation of fecal bacteria for flow cytometry.** One-half gram of feces was suspended in 4.5 ml filtered (0.22 μm) PBS, homogenized on a Vortex mixer for 1 min, and centrifuged at low speed (35g, 20 min) to separate larger fecal particles from bacteria. From each supernatant, 20 μl (containing about 10⁹ bacteria, as determined by direct microscopic clump counts (9)) was washed once in 1 ml fil-
tered PBS and centrifuged at 8,000g for 10 min to remove nonbound fecal IgA. The pellet was resuspended in 60 µl of BSA/PBS (1% w/v) or in FITC-labeled goat F(ab')2 antihuman IgA (1:100 in BSA/PBS (1% w/v)). Suspensions were incubated for 30 min at room temperature. One milliliter PBS was added and mixed prior to centrifugation (8,000g, 10 min). This wash procedure was repeated once. Finally, the bacterial pellet was resuspended in 500 µl PBS, added to 20 µl PI (4 mg/liter final concentration was found to be optimal), stored on ice in the dark, and analyzed within 2 h.

In order to determine the specificity of the FITC-F(ab')2 goat anti-Hu-IgA, a FITC-F(ab')2 goat antimouse-IgM was added to human fecal bacterial suspensions. Further processing was identical to the procedure described above.

**Preparation of pure cultured bacteria for flow cytometry.** Pure cultured bacteria were washed once with PBS, adjusted to the optimal concentration (10^8 bacteria/ml) in 500 µl, and added to 20 µl PI. (Several PI concentrations were tested (100, 20, 4, 0.8 and 0.16 mg/liter), and 4 mg/liter was found to be the optimal concentration. Higher PI concentrations resulted in an increased background fluorescence without detection of more bacteria, whereas with lower PI concentrations less bacteria was detected.) The bacterial suspensions were stored on ice in the dark and analyzed within 2 h. For vital pure cultured aerobic bacteria, the discriminator was set on FSC. Special precautions were taken to prevent environmental contamination by these bacteria.

**Computer Image Analysis**

**Instrumentation.** The instrumentation has been described in detail elsewhere (1,29). Briefly, we used a microscope equipped with a phase-contrast, mercury vapor lamp and a BP490 filter block (transmission of 490–750 nm). A high-resolution CCD video camera was mounted on top of the microscope and connected with a computer. An exposure-control expansion board enabled us to record long-exposure images (4 s). Fluorescence measured by each pixel of the video camera was calibrated using a uniformly fluorescent uranyl glass slide (Schott, Mainz, Germany) and was therefore expressed in uranyl units (uU). Fluorescence quantification was done with fluorescein quantitation kits (Quantum 24 and Quantum 25). A 2D-surface area threshold was set on 0.15 µm^2, i.e., small objects not believed to be bacteria.

**Actual recording.** To record the fluorescence of each object in the microscopic field of view, it is necessary to acquire two images: 1) with morphological information (phase contrast) and 2) with fluorescence information. For each object, the average level of fluorescence within the object as well as its exact morphology and surface area were computed. All measurements were performed with at least 1,000 objects per sample, and the median and third quartile of fluorescence as well as the median bacterial surface area were determined.

**Isolation and preparation of fecal bacteria for image processing.** The procedure is in part analogous to the sample preparation procedure used for flow cytometry (1). One-half gram of feces was suspended in 4.5 ml of demineralized water, homogenized on a vortex mixer for 1 min, and centrifuged at low speed (35g, 20 min). The bacteria in the supernatant were diluted to a 2% suspension in demineralized water with 0.5% Tween 80 (Merck, Darmstadt, Germany), and 10 µl was pipetted into a well of three different degreased 12 well slides (Immunocor, Limogenes, France) that previously had been coated with a 10% poly-L-lysine solution (Sigma Diagnostics) in order to ensure optimal adhesion of the fecal bacteria to the slide. After drying and fixation in acetone for 10 min, gentle washing (PBS, 5 min), and again drying, slides were stored at −20°C before use. After thawing, 20 µl of FITC-labeled goat F(ab')2 antihuman IgA (1% w/v) in BSA/PBS (1% w/v) or 20 µl of BSA/PBS (1% w/v) was pipetted into each well. After incubation for 30 min in a moist chamber at 20°C in the dark, slides were washed gently three times in PBS, embedded in mounting fluid (glycerol/Tris buffer w/v 1:1), covered with a coverslide, and stored in a moist chamber at 4°C in the dark for at least 2 h and maximally 72 h. A third slide was stained with PI (20 µl, 100 mg/liter PBS) and analyzed within 2 h. Pure cultured bacteria were processed as for flow cytometry, and image analysis recording was identical to the procedure for fecal suspensions.

**Statistical Analysis**

Spearman rank correlation coefficients were computed to determine the relations between variables of fluorescence, FSC, and surface area. Two-tailed probabilities are presented. Simple linear regression analysis was performed in order to determine the best fitted line between variables. The coefficient of variation of six separate analyses of one fecal sample was used to describe the intraassay variation of flow cytometry.

**RESULTS**

**Bacterial Size Calibration**

In flow cytometry, forward scatter (FSC) corresponds to cell volume (11,21). However, with image analysis, the 2D surface area of recorded bacteria is determined. Therefore, we determined the relation between FSC and 2D bacterial surface area by measurement of seven pure cultures of human feces-derived aerobic and anaerobic bacteria with flow cytometry and image analysis. There was a considerable variation in FSC within a pure culture [coefficient of variation (CV) 35–100%]. However, FSC mean and surface area median were correlated (p = 0.964, P < 0.01; Fig. 1). The smallest bacterial species measured, a *Pseudomonas* spp., had a median surface area of 0.55 µm^2 and a mean FSC of 23.
FLOW CYTOMETRY OF FECAL ANAEROBIC BACTERIA

Fluorescence Quantitation

We aimed to attribute molecules of equivalent soluble fluorochrome (MESF) values to the FITC-fluorescence as measured via flow cytometry (25). Hence we analyzed, by logarithmic recording, latex particles with defined MESF values as well as “nonfluorescent” particles of various sizes (8.6, 6, and 0.79 μm). FITC-fluorescence values were linearly correlated with MESF values (Fig. 2; \( r = 0.975, P = 0.0001 \)). Logarithmic fluorescence recording tends to be less accurate than linear recording at very high or low fluorescence values. Since for bacterial analysis we use logarithmic fluorescence recording, we intended to quantify this inaccuracy by analysis of the latex particles with both types of recording. Logarithmically and linearly recorded fluorescence values were similar for all fluorescent particles (\( r = 0.99, P = 0.0001 \)). However, many fecal anaerobic bacteria have very low fluorescence, and there are no calibration particles with such low MESF values. Therefore, we analyzed “nonfluorescent” particles of different sizes as well by both types of recording, and their “fluorescence” increased with their size. Assuming that the measured linear mean in this regions with extremely low fluorescence is still accurate, we extrapolated the relation between linearly recorded FITC-fluorescence of nonfluorescent latex particles and MESF (Fig. 2). The extrapolated fluorescence values of the two smallest particles were 120 MESF and 250 MESF. At these extremely low fluorescence values, logarithmically recorded “fluorescence” was 50% lower than linearly recorded fluorescence (Fig. 2). This suggests that as few as about 300 FITC molecules can be detected with logarithmic recording.

In order to attribute MESF to fluorescence values determined by image analysis, fluorescein quantitation particles were measured by image analysis as well. Fluorescence recorded by our image analysis system is expressed in uranyl units (uU), i.e., fluorescence per square micrometer. Therefore, MESF values were corrected for the calculated 2D-surface area of the particles. These corrected MESF values were linearly correlated with the fluorescence expressed in uU (1 uU = 34 × 10^3 MESF/μm^2).

Elimination of Large Aggregates

Fecal suspensions contain large particles, apart from single bacteria. Despite low-speed centrifugation during sample preparation, large particles were still observed during flow cytometry measurement. In order to determine the FSC above which no single bacteria were present, the 2D-surface area of fecal bacteria present in samples of 22 healthy human volunteers were measured via computer image analysis. Objects with a 2D-surface area larger than 5 μm^2 (less than 1% of all objects, since only images with few aggregates were recorded) were nearly all composed of aggregated bacteria (data not shown). Extrapolation of the relation between surface area and FSC shows that 5 μm^2 corresponds with FSC = 1,000 (Fig. 1). Therefore, all further flow cytometry analyses were performed with a gate set on FSC < 1,000. Thereby 6% of the events were excluded. In order to check whether events with FSC > 1,000 were aggregates, a sorting experiment was per-
formed with a gate on FSC >1,000. Sorted objects visually evaluated with phase contrast microscopy all appeared to be aggregates. However, 8% of all objects with a surface area of <5 μm² appeared to be aggregates of smaller bacteria and could therefore not be excluded by criteria based on bacterial size.

A possible relation between side scatter (SSC) and bacterial morphology was investigated via flow cytometry sorting of bacteria with high SSC. Visual evaluation of the sorted events with phase contrast microscopy showed that events with very high SSC were bacteria with an irregular cytoplasmic structure. No relation with aggregation was found.

**Only PI⁺ Events Were Analyzed by Flow Cytometry**

For flow cytometry analysis of eukaryotic cells, a discriminator is usually set on FSC. However, the FSC of a proportion of fecal bacteria is too low to be analyzed with a discriminator set on low FSC (Fig. 3a,b). We therefore stained all fecal bacterial suspensions with PI, a fluorescent stain for double-stranded DNA/RNA. Since anaerobic bacteria will have died during our sample preparation procedure, they will have become permeable for PI. With a discriminator set on PI fluorescence, it was possible to analyze events with very low FSC (Fig. 3a).

A second advantage of a discriminator set on PI fluorescence is exclusion of PI⁻ nonbacterial compounds (e.g., cellular debris, mucus) and bacterial fragments present in washed fecal bacterial suspensions. To make sure that the excluded PI⁻ events were indeed fragments or objects of nonbacterial origin, their morphology was determined by flow cytometry sorting (with a discriminator set on FSC). Only a small proportion (20%) of all events was PI⁻ (Fig. 3b). Visual evaluation of the sorted PI⁻ events by phase contrast microscopy was performed. Besides large amounts of small objects (presumably bacterial fragments), irregular clearly nonbacterial objects (presumably mucus fragments and undigested dietary compounds) were seen.

A second experiment to determine the morphology of PI⁻ objects was performed by image analysis of PI-stained slides with bacterial suspensions of all fecal samples. The PI-fluorescence distribution showed a distinct nonstained peak (PI⁻). The median percentage of PI⁻ objects was 40% (range 22–65%), with a median 2D-surface area of 0.46 μm². With this image analysis system, it is possible to plot bacteria according to two independent morphology variables (1). Morphologically, PI⁻ objects were largely concentrated in the region of small cocoid objects, which was scarcely populated by PI⁺ objects. With our image analysis system, it is not yet possible to measure FITC at the same time as PI. In order to eliminate many PI⁻ objects from further analysis by image analysis software, we excluded all small cocoid objects (18%, of which 80% were PI⁻) by morphological criteria. The median percentage of PI⁻ objects remaining was 28%.

**Size of Fecal Anaerobic Bacteria**

Fecal bacteria form a morphological heterogenous population (Fig. 4). In order to determine the normal variation in distribution of fecal bacterial size, we analyzed fecal bacterial suspensions of 22 healthy human volunteers via both flow cytometry and image analysis. There was a considerable variation of FSC within samples as expressed by a CV of 110–159% (Fig. 3). The median FSC of all samples was 129 (range 79–183), corresponding to a 2D-surface area of 1.0 μm² (Fig. 1). The mean FSC intraassay CV (9%) was lower than the mean FSC CV of all samples (19%). Most (80%) PI⁺ events had a FSC <200, corresponding to a 2D-surface area of <1.5 μm².

For determination of the median bacterial 2D-surface area, PI-stained slides were measured by image analysis, and PI⁺ objects were evaluated. The median bacterial 2D-surface area of all fecal samples was 1.07

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In Vivo IgA Coating of Fecal Anaerobic Bacteria

One of the aims of this study was to analyze the technical possibility of measurement of in vivo IgA coating of anaerobic bacteria present in fecal suspensions by flow cytometry. Therefore, we analyzed fecal bacterial suspensions stained with FITC-F(ab')$_2$ anti-Hu-IgA as well as nonstained samples by flow cytometry. All samples were stained with PI as well, and the discriminator was set on PI fluorescence.

Fluorescence of nonstained bacteria as measured with FITC filter settings was present only in the lower channels (Fig. 5). The fluorescence distributions of the nonstained samples ($n = 22$) were similar, with a median value of 0.24 (range 0.17–0.27, CV = 6%), corresponding to $2 \times 10^2$ MESF. To exclude the possibility that the recorded fluorescence of nonstained samples was dominated by photomultiplier background noise, we analyzed nonfluorescent latex particles of bacterial size (0.79 μm). Their mean fluorescence value was 0.142, and their fluorescence distribution was smaller than that of all nonstained fecal suspensions. Thus only a portion of the fluorescence of nonstained samples consists of background noise. To determine the specificity of the measurement of "in vivo" IgA coating of fecal bacteria, fecal samples were stained with a nonsense FITC-F(ab')$_2$-antimouse IgM. No nonspecific staining was detected.

All samples were found to contain IgA-coated bacteria, and there was not a clear discrimination between fluorescence values of stained and nonstained bacteria (Fig. 5). However, in one sample a separate, strongly IgA-coated bacterial population was seen (Fig. 6). In order to estimate the percentage of fecal bacteria coated with IgA and their levels of fluorescence, we performed match region subtraction (22) with Immuno-4 software. With this method, a fluorescence histogram of nonstained bacteria is matched over a region (match region) with the corresponding histogram of stained bacteria, its height (size) is adjusted, and thereafter both are subtracted (Fig. 6). The median percentage of stained bacteria was 45% (range 24–74%, CV = 28%), and their median fluorescence value was 2.33 (range 0.99–4.74, CV = 38%) corresponding to 1,300 MESF. The intraassay variations were 9% (percentage of stained bacteria) and 7% (mean fluorescence value). Within the samples, there was not a clear relation between FSC and fluorescence of bacteria labeled with FITC-anti-IgA (Fig. 5). Neither was there a relation between the mean FSC and the percentage of stained bacteria of all samples, nor between the mean FSC and the mean specific fluorescence.

In order to validate flow cytometry data, all fecal samples were analyzed via image analysis as well. Since total numbers of bacteria recorded via image analysis were low and no histogram subtraction software was available, only the median and third quartile (Q3) of fluorescence were calculated. The median of all median fluorescence values of nonstained samples was 0.015 uU (range 0.008–0.023, CV = 26%) corresponding to $5 \times 10^2$ MESF/μm$^2$. For the Q3 of fluorescence, the median value was 0.022 uU. Also with image analysis, all samples were found to contain IgA-coated objects, and there was not a clear discrimination between fluorescence distributions of stained and nonstained objects. In order to estimate the specific fluorescence (i.e., fluorescence due to labeling with FITC-anti-IgA) of each sample, the median (and Q3 of) fluorescence of nonstained bacteria was subtracted from the median (and Q3 of) fluorescence of stained bacteria. For all samples, the median specific fluorescence was 0.011 uU (range 0.002–0.025, CV = 43%) corresponding to $4 \times 10^4$ MESF/μm$^2$. The median value of all Q3 of specific fluorescence was 0.029 uU.

Fluorescence data of bacteria labeled with FITC-anti-IgA obtained by both methods were correlated. The mean fluorescence of stained bacteria as determined by flow cytometry correlated significantly with the Q3 of specific fluorescence obtained by image analysis ($r = 0.54, P < 0.05$; Fig. 7). In contrast to the Q3, the median specific fluorescence (image analysis) did not correlate with flow cytometry data.

Strongly fluorescent bacteria within a mixed population such as fecal flora should be recognized by both systems. Fortunately, one fecal sample contained a separate small population (2%) of strongly FITC-anti-IgA-labeled bacteria with a mean FSC of 180 (Fig. 6). Morphology of these bacteria was determined by image analysis as well as by flow cytometry sorting. The most
FIG. 5. Typical example of fluorescence distributions of nonstained bacteria (overlay; solid line) and bacteria labeled with FITC-anti-IgA (dotted line) as recorded by flow cytometry. The fluorescence histogram of nonstained bacteria was matched with the histogram of stained bacteria and subtracted by match region subtraction. The matched region (A), the region in which the percentage of stained bacteria is calculated (C), and the resulting fluorescence histogram (positives) are shown.

FIG. 6. FSC vs. FITC fluorescence distribution of a fecal bacterial suspension stained for IgA. This sample contained a separate strongly IgA-coated population, which was sorted via flow cytometry (gate N).

Fluorescent bacteria recorded by image analysis were coccoid rods with a median surface area of 1.3 μm². Flow cytometry-sorted bacteria were visually evaluated and were morphologically identical to the coccoid rods recorded by image analysis.

DISCUSSION
Flow Cytometry Analysis of Noncultured Fecal Anaerobic Bacteria

In the present paper, we describe a new rapid flow cytometry-based method to analyze noncultured anaerobic bacteria present in human fecal suspensions. Major problems in our approach appeared to be the small size of some fecal bacteria, the presence of nonbacterial objects and bacterial fragments, as well as the presence of larger objects formed by bacterial aggregates. Since aerobic bacteria make up less than 0.1% of the fecal flora, their contribution to the data will be negligible (15).

A discriminator set on FSC, as is commonly used for eukaryotic analysis, is sufficient to analyze suspensions of larger bacteria without interference of background noise (2,6,14,18). Our fecal bacterial suspensions, however, appeared to contain small bacteria as well, with an FSC at a level at which background noise is present. As was described by others for aerobic bac-
Fecal bacterial suspensions contain large particles, apart from single bacteria. However, despite low-speed centrifugation during sample preparation, large particles were still observed during flow cytometry analysis. For this reason, we used size as an additional selection criterion. With a gate on FSC <1,000, all single bacteria were analyzed. However, 8% of all particles with an FSC <1,000 were agglutinated small bacteria. In conclusion, with a discriminator set on PI fluorescence and exclusion of events with high FSC, most if not all analyzed events are intact predominantly single fecal anaerobic bacteria.

Size of Anaerobic Fecal Bacteria

To our knowledge, no data on the size of bacteria present in fecal suspensions have been published as yet. Therefore, fecal samples from 22 healthy volunteers were analyzed via flow cytometry, and data were validated with analysis of the same samples by computer image analysis as well. Fecal flora consists of a heterogenous population of possibly as many as 400 different species of anaerobic bacteria (8). In fecal suspensions, each species is present in low concentrations of <3% (7). This heterogeneity was reflected in the large CV of the FSC distributions within a sample, compared to pure cultures. The majority of the analyzed fecal bacteria were small, with an FSC corresponding to a 2D-surface area of <1.5 μm². This surface area is smaller than that of pure cultures of E. coli or Klebsiella spp. but larger than that of Pseudomonas spp. There was an interindividual variation in mean FSC (CV = 19%). However, since no longitudinal study was performed, it is not possible to ascribe this interindividual variation in FSC to stable interindividual differences in fecal flora composition, for which some evidence can be found in the literature (7,15). In conclusion, fecal suspensions contain a heterogenous bacterial population, with large variation in bacterial size. However, most fecal bacteria are small, with a 2D-surface area of <1.5 μm².

Quantitation of Low FITC Fluorescence Values

FITC fluorescence as measured with flow cytometry was quantitated with fluorescein-coated particles in molecules of equivalent soluble fluorochrome (MESF), and there was an excellent linear correlation between MESF and FITC fluorescence. In order to attribute MESF to the fluorescence levels comparable to those of anaerobic bacteria labeled with FITC-anti-IgA, we had to extrapolate the linear relation between MESF- and FITC- fluorescence of “nonfluorescent” latex particles. Here, the lower detection limit of logarithmically recorded fluorescence of our flow cytometer was found to be as low as 3 x 10² MESF, assuming that the photomultiplier was perfectly linear in this fluorescence region. Deviations from linearity will cause an increase in this MESF value.

In Vivo IgA Coating of Fecal Anaerobic Bacteria

One of the aims of this study was to determine the technical possibility of measurement via flow cytometry of in vivo IgA coating of anaerobic bacteria present in fecal suspensions. In the gut lumen, IgA is secreted by the intestinal mucosa in large amounts, and specificity for intestinal anaerobic bacteria is present (17). All fecal samples were found to contain IgA-coated as well as non-IgA-coated bacteria. The mean fluorescence of non-IgA-coated bacteria was 2 x 10² MESF and presumably was composed of photomultiplier background noise and autofluorescence.

In most samples, there was no clear discrimination between fluorescence distributions of stained and nonstained bacteria. For this reason, we performed match region subtraction by which fluorescence histograms of nonstained samples are matched over a region with histograms of corresponding stained samples and subtracted. This subtraction method was found to be very accurate for analysis of overlapping distributions of stained and control samples, compared to simple subtraction or threshold analysis, and nearly as good as curve fitting (22). The median percentage of IgA-coated bacteria was 45%, with a mean fluorescence of 1,300 MESF. However, the “mean” of logarithmic recorded parameters as presented by ELITE software is not a true mean. The mean channel number of all events is determined, and the corresponding parameter LOG value is calculated and presented as the mean. This “mean” will be lower than the true mean. An advantage is, however, that the “mean” will be more robust and therefore less susceptible to small changes of events with high parameter values. Amplification of the staining signal, for instance, by biotin labeling of the anti-IgA polyclonal, is a second approach to increase the sensitivity of estimation of the percentage of stained bacteria. However, this was beyond the scope of this study.

In order to validate measurement of in vivo IgA coating by flow cytometry, we determined in vivo IgA coating with computer image analysis as well. There was a
significant correlation between the mean fluorescence (flow cytometry) of bacteria labeled with FITC-anti-IgA and the third quartile of specific fluorescence as recorded by image analysis. The absence of a correlation between the median of specific fluorescence (image analysis) and flow cytometry data is probably partly due to a larger relative contribution of the auto-fluorescence to image analysis data due to less selective fluorescence filters. These increased auto-fluorescence values resulted in a decreased relative difference between the median fluorescence of nonstained bacteria (auto-fluorescence) and the median fluorescence of bacteria labeled with FITC-anti-IgA and therefore a larger influence of statistical variation. Furthermore, a considerable but variable percentage of the image analysis data consisted of PI- objects. Finally, the "mean" as presented based on ELITE software is presumably closer to the third quartile than to the median value.

In conclusion, with flow cytometry it is possible to measure in vivo IgA coating present on fecal bacteria with high sensitivity.

**Comparison of Flow Cytometry With Image Analysis**

In order to compare flow cytometry with our image analysis system (1,10), analogous parameters of bacterial size and fluorescence were measured for pure cultures and all 22 human fecal samples. In contrast to the case with flow cytometry, with our image analysis system it is not yet possible to measure two different fluorescent stains at the same time, i.e., FITC together with PI. Consequently, PI- objects could be only partially eliminated from further evaluation with morphological criteria. We found a linear relation between FSC and bacterial 2D-surface area as determined with analysis of several pure cultures by flow cytometry as well as image analysis. Robertson and Button (21), however, found a linear relation between FSC and bacterial volume. Presumably, in this small 2D-surface area region, there is an approximately linear relation between bacterial 2D-surface area and bacterial cell volume.

In contrast to flow cytometry, image analysis records morphology in addition to bacterial size. Furthermore, the threshold for accurate measurement of bacterial size is lower for image analysis (0.15 μm²) than for flow cytometry (0.5 μm²). However, flow cytometry acquisition rate is 1,000-fold higher compared to image analysis. Flow cytometry, furthermore, was more effective in the reduction of auto-fluorescence, could effectively eliminate PI- events, and offered match region subtraction software. For these reasons, flow cytometry was more sensitive for measurement of low levels of FITC fluorescence than image analysis. Nevertheless, as was discussed above, there was a significant correlation between specific fluorescence values as determined by both methods. Further evidence for a comparable determination of higher levels of specific fluorescence on bacteria was obtained via analysis of the morphology of a small population of strongly FITC-anti-IgA-labeled bacteria by both methods. Visual evaluation of this population obtained by flow cytometry sorting revealed a morphology identical to that of the strongest fluorescent bacteria as determined by image analysis.

**CONCLUSIONS**

In conclusion, with flow cytometry it is possible to analyze intact single fecal bacteria within highly heterogeneous bacterial populations and without interference of contaminating nonbacterial compounds, bacterial fragments, and aggregates. Furthermore, it is possible to measure low levels of bacterial in vivo IgA coating by means of immunofluorescence.

In comparison with image analysis, flow cytometry is more sensitive for measurement of low levels of specific fluorescence and has a very high acquisition rate. However, image analysis records bacterial morphology and has a lower 2D-surface area threshold. Despite these differences, parameters of bacterial size and specific fluorescence as measured using both methods were correlated. This validates both methods.

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**LITERATURE CITED**

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