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The interaction between the mucosal immune system and the commensal microflora of the colon

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Chapter 2

Direct flow cytometry of anaerobic bacteria in human faeces

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Cytometry 1994;16:270-9.

Abstract

Background. In the present study we describe a flow cytometry method for analysis of noncultured anaerobic bacteria present in human faecal suspensions. Non-bacterial faecal 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 comprise over 99.9% of all faecal 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 faecal anaerobic bacteria as well as their bacterial size.

Methods. Faecal samples of 22 healthy human volunteers were analysed. The fluorescence distribution of IgA coated bacteria labeled with 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.

Results. The median bacterial 2-dimensional surface area was $1.0 \mu\text{m}^2$. To validate flow cytometry data, all samples were analysed by an image analysis system as well.

Conclusion. With this new method a rapid evaluation of faecal flora with high sensitivity for specific FITC-fluorescence is possible without culturing.

Introduction

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 analyse bacterial populations. In this field, the focus has been on bacterial pure cultures¹⁻⁵, though in some studies noncultured mixed bacterial populations like aquatic bacteria were characterised^{6,7}. Flow cytometry may be a potential technique to analyse faecal flora as well. However, no reports on this subject have been published yet.

There is a growing interest in analysis and characterisation of the anaerobic intestinal flora. Faecal anaerobic bacteria may play a role in the pathogenesis of diseases like graft-versus-host disease^{8,9}, inflammatory bowel disease^{10,11} and bacterial cell wall induced arthritis in rats¹². Furthermore, intestinal anaerobic bacteria may inhibit colonisation of the intestinal tract by potentially pathogenic and pathogenic bacteria and therewith prevent infections¹³.

The human colon harbors about 10^{11} bacteria per gram contents. It is important to realise 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 an individually characteristic composition¹⁴⁻¹⁶.

This composition may be influenced by IgA that is secreted in large amounts into the intestinal lumen. Potentially pathogenic aerobic bacteria like *Enterobacteriaceae spp* (e.g. *Escherichia coli*) comprise less than 0.1% of the colonic flora.

Faecal anaerobic bacteria are difficult to study. Culturing and identifying anaerobic bacteria by biochemical properties is 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^{14, 17-20}. Therefore, we aim to examine noncultured human faecal anaerobic bacteria. A few years ago we have developed a phasecontrast-fluorescence microscope based computer image analysis system that enables us to record morphology and immuno-fluorescence labeling of each faecal bacterium separately²¹⁻²³. This image analysis method, however, is quite laborious.

In the present study we describe a new rapid method to characterise the anaerobic faecal flora by flow cytometry. Faecal samples of 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 faecal anaerobic bacteria. To validate flow cytometry data, all samples were analysed by a computer image analysis system as well.

Materials and methods

Volunteers

Twenty two healthy volunteers, 13 male and 9 female, aged 21-61 years (median 32 years), provided a faecal sample. Exclusion criteria were: immunocompromised conditions (corticosteroids, diabetes, etc.), antibiotic use less than two weeks before sampling, diarrhoea and pregnancy. Each faecal sample was divided into 12 portions of 0.5 gram, frozen within three hours after defecation and stored until use at -20°C.

Laboratory analysis

Pure cultures: Pure cultures of human faeces derived *Bacteroides fragilis*, *Fusobacterium spp.*, *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 anti-human IgA (F/P ratio = 2.0) from Kallestad (Austin, TX), FITC-labeled goat F(ab')₂ anti-mouse IgM (F/P ratio = 3.2) from Protos immunoresearch (San Francisco, Cal), BSA (fraction V) from Boehringer Mannheim (Mannheim, Germany), and propidium iodide (PI) from Sigma (St Louis, MO) were used.

Study design: Faecal samples and suspensions of different pure cultured bacteria were analysed by flow cytometry as well as by image analysis. *In vivo* IgA coating of anaerobic bacteria was determined by staining faecal suspensions with FITC-F(ab')₂ anti-human IgA and subtraction of background-fluorescence as measured in nonstained suspensions. In order to determine the intra assay variation of measurement of bacterial *in vivo* IgA coating, replicate analyses of a single faecal sample were included in each series of measurements.

Flow cytometry

Flow cytometry was performed with an EPICS - ELITE (Coulter - Electronics, Hialeah, FL). Sample excitation was done by an Argon laser operating at 15 mW and 488 nm. Filtersettings were 525 BP for FITC, 550 LP and 630 BP for measurement of PI. Acquisition and data analysis were done with standard ELITE software comprising the Immuno-4 program to determine the percentage of stained events. 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., NC, USA). To determine the level of background noise we used plain microspheres (Polysciences, Inc., Warrington, PA) 0.79 µm in diameter, which were assumed to have no fluorescence. For bacterial measurements the discriminator was set on propidium iodide (PI) fluorescence as a specific probe for bacteria. The discriminator value was determined by a filtered bacteria free (0.22 µm Millipore., Molsheim, France) solution of PI / PBS (4 mg/l) 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/l) at a level with minimal background noise.

Actual analysis: Of each sample a portion incubated with PBS (background fluorescence) and a portion incubated with FITC-labeled goat F(ab')₂ anti-human IgA were analysed. Both measurements were performed with 10,000 events, at a flow rate of 1000 - 1500 events / sec. Data were stored in listmode on disc. The fluorescence was recorded logarithmically, FSC linearly. The mean fluorescence (logarithmic scale) was the fluorescence value corresponding with the calculated mean channelnumber (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 > 1000 and on FSC < 1000 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 by microscopy and computer image analysis.

Isolation and preparation of faecal bacteria for flow cytometry: Half a gram of faeces was suspended in 4.5 ml filtered (0.22 μm) PBS, homogenized on a Vortex mixer during 1 min. and centrifuged at low speed (35g, 20 min) to separate larger faecal particles from bacteria. Of each supernatant 20 μl (containing about 10^8 bacteria, as was determined by Direct Microscopic Clump Counts²⁹) was washed once in 1 ml of filtered PBS and centrifuged at 8000g for 10 min to remove non-bound faecal IgA. The pellet was resuspended in 60 μl of BSA / PBS (1% w/v) or in FITC-labeled goat F(ab)₂ anti-human IgA (1:100 in BSA / PBS (1% w/v)). Suspensions were incubated for 30 min at room temperature. One ml of PBS was added and mixed prior to centrifugation (8000g, 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/l (final concentration) was found to be optimal), stored on ice in the dark and analysed within two hours.

In order to determine the specificity of the FITC-F(ab')₂ goat anti-Hu-IgA, a FITC-F(ab')₂ goat anti-mouse-IgM was added to human faecal 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 bacterial concentration (10^8 bacteria / ml) in 500 μl , added to 20 μl PI (final concentration: 4 mg /l), stored on ice in the dark and analysed within two hours. 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

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 sec.). 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 (QuantumTM 24 and QuantumTM 25). A 2D-surface area threshold was set on 0.15 μm^2 , i.e. small objects not believed to be bacteria. To record the fluorescence of each object in the microscopic field of view, it is necessary to acquire two images (a) with morphological information (phasecontrast), and (b) 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 1000 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 faecal bacteria for image processing: The procedure is in part analogous to the sample preparation procedure used for flow cytometry¹. Half a gram of faeces was suspended in 4.5 ml of demineralised water, homogenized on a Vortex mixer during 1 min. and centrifuged at low speed (35g, 20 min). The bacteria in the supernatant were diluted to a 2% suspension in demineralised water with 0.5% Tween 80 (Merck, Germany) and 10 μl was pipetted into a well of three different degreased twelve well slides (Immunocor, France) which previously had been coated with a 10% poly-l-lysine solution (Sigma Diagnostics, St. Louis., USA) in order to ensure optimal adhesion of the faecal bacteria to the slide. After drying and fixation in acetone during 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 anti-human IgA (1% (v/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 v/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 max. 72 h. A third slide was stained with PI (20 μ l, 100 mg / l PBS) and analysed within two hours. Pure cultured bacteria were processed as for flow cytometry and image analysis recording was identical to the procedure for faecal suspensions.

Statistics

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 faecal sample was used to describe the intra-assay variation of flow cytometry.

Results

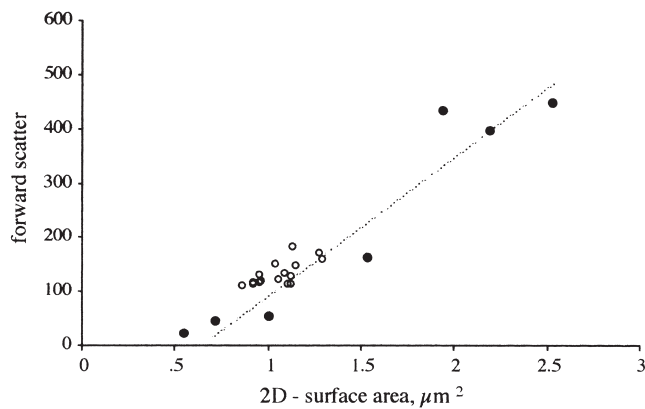


Figure 1. Relation between median 2D-surface area (image analysis) and mean forward scatter (flow cytometry) for pure cultures (closed circles, n=7) and faecal samples (open circles, n=22). Pure cultured bacteria, ordered according to their surface area (smallest surface area first), were *Pseudomonas* spp., *Bacillus fragilis*, *Fusobacterium* spp., *Salmonella* spp., *Klebsiella* spp., *Clostridium perfringens* and *Escherichia coli*. For pure culture data, the best fitted line as calculated by linear regression analysis is shown.

Bacterial size calibration

In flow cytometry forward scatter (FSC) corresponds to cell-volume^{6,24}. However, with image analysis the 2 Dimensional (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 faeces 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 ($\rho = 0.964$, $p < 0.01$, fig 1). The smallest bacterial species measured, a *Pseudomonas* spp., had a median surface area of $0.55 \mu\text{m}^2$ and a mean FSC of 23.

Fluorescence quantitation

We aimed to attribute Molecules of Equivalent Soluble Fluorochrome (MESF) values to the FITC-fluorescence as measured by flow cytometry²⁵. Hence we analysed by logarithmic recording, latexparticles with defined MESF values as well as ‘non-fluorescent’ particles of various sizes ($8.6 \mu\text{m}$, $6 \mu\text{m}$, $0.79 \mu\text{m}$). 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 latexparticles with both types of recording. Logarithmically and linearly recorded fluorescence values were similar for all fluorescent particles ($r=1.0$, $p=0.0001$). However, many faecal anaerobic bacteria have very low fluorescence and there are no calibration particles with such low MESF values. Therefore, we analysed ‘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 latexparticles 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 by logarithmic recording.

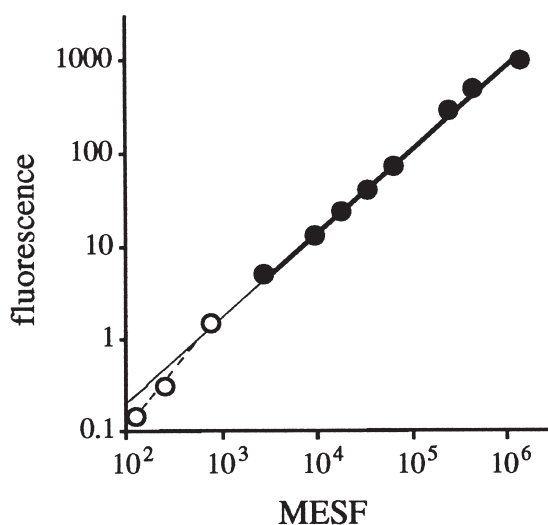


Figure 2. Relation between MESF and fluorescence as determined by both logarithmically and linearly recording of FITC-fluorescence quantitation particles by flow cytometry (closed circles, only linearly recorded data are visualised). The best fitted line as calculated by linear regression analysis is shown. MESF values of linearly recorded nonfluorescent particles were extrapolated assuming that the photomultiplier was perfectly linear in this fluorescence region (continuous line). Extrapolated MESF values of logarithmically recorded nonfluorescent particles were determined with respect to the linearly recorded values (open circles, dashed line).

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 μm^2 . 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 ($1 \text{ uU} = 34 \times 10^3 \text{ MESF} / \mu\text{m}^2$).

Elimination of large aggregates

Faecal 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 faecal bacteria present in samples of 22 healthy human volunteers were measured with computer image analysis. Objects with a 2D-surface area larger than $5 \mu\text{m}^2$ (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 $5 \mu\text{m}^2$ corresponds with $\text{FSC} = 1000$ (fig 1). Therefore, all further flow cytometry analysis were performed with a gate set on $\text{FSC} < 1000$. Therewith 6% of the events were excluded. In order to check whether events with $\text{FSC} > 1000$ were aggregates, a sorting experiment was performed with a gate on $\text{FSC} > 1000$. Sorted objects visually evaluated by phasecontrast microscopy, all appeared to be aggregates. However, 8% of all objects with a surface area $< 5 \mu\text{m}^2$ 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 by flow cytometry sorting of bacteria with high SSC. Visual evaluation of the sorted events by phasecontrast microscopy showed that events with very high SSC were bacteria with an irregular cytoplasmatic structure. No relation with aggregation was found.

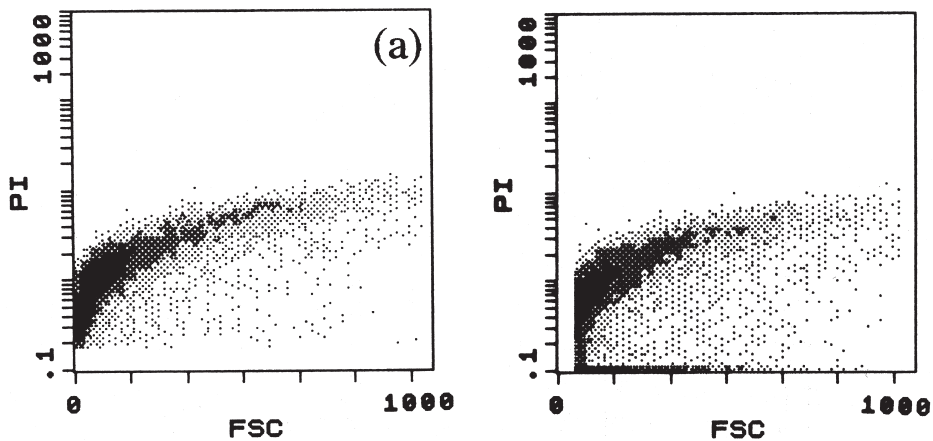


Figure 3. Typical forward scatter (FSC) versus propidium iodide (PI) fluorescence distribution of a PI-stained faeces derived bacterial suspension. A discriminator was set on PI-fluorescence (3a) or FSC (3b) at a level with minimal background noise.

Only Propidium Iodide positive events were analysed by flow cytometry

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

A second advantage of a discriminator set on PI-fluorescence is exclusion of PI⁻ non-bacterial compounds (e.g. cellular debris, mucus) and bacterial fragments present in washed faecal bacterial suspensions. To make sure that the excluded PI⁻ events were indeed fragments or objects of non-bacterial 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 phasecontrast microscopy was performed. Besides large amounts of small objects, presumably bacterial fragments, irregular clearly non-bacterial 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 faecal samples. The PI-fluorescence distribution showed a distinct non-stained peak (PI⁻). The median percentage PI⁻ objects was 40% (range 22-65%) with a median 2D-surface area of 0.46 μm^2 . 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 coccoid 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 coccoid objects (18%, of which were 80% PI⁻) by morphological criteria. The median percentage PI⁻ objects remaining was 28%.

Size of faecal anaerobic bacteria

Faecal bacteria form a morphological heterogenous population. In order to determine the normal variation in distribution of faecal bacterial size, we analysed faecal bacterial suspensions of 22 healthy human volunteers both by 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 with a 2D-surface area of 1.0 μm^2 (fig 1). The mean FSC intra-assay CV (9%) was lower than the mean FSC CV of all samples (19 %). Most (80%) PI⁺ events had a FSC < 200, corresponding with a 2D-surface area of < 1.5 μm^2 .

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 faecal samples was 1.07 μm^2 (range 0.78 - 1.30, CV = 11%). Of each sample about 80% of the PI⁺ objects had a surface area < 1.5 μm^2 which is comparable to flow cytometry. The correlation between median bacterial 2D-surface area and mean FSC of all samples was comparable to pure cultures as is shown in fig 1 ($\rho = 0.68$, $p < 0.01$).

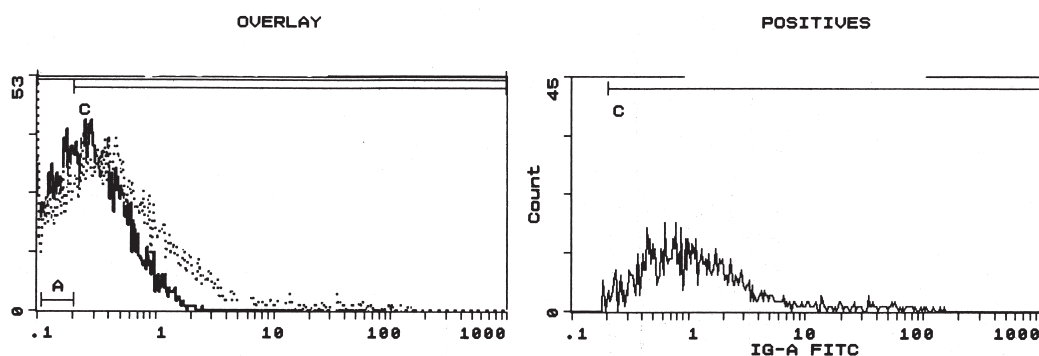


Figure 4. Typical example of fluorescence distributions of nonstained bacteria (overlay, continuous line) and bacteria labeled with FITC-anti-IgA (dotted line) as recorded by flow cytometry. The fluorescence histogram of nonstained bacteria was matched with the histogram of stained bacteria and subtracted 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 (overlay) are shown.

***In vivo* IgA coating of faecal anaerobic bacteria**

One of the aims of this study was to analyse the technical possibility of measurement of *in vivo* IgA coating of anaerobic bacteria present in faecal suspensions by flow cytometry. Therefore, we analysed faecal 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 only present in the lower channels (fig 4). 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 with 2×10^2 MESF. To determine the contribution of photomultiplier background noise to these extremely low fluorescence values, we analysed nonfluorescent latex-particles of bacterial size (0.79 μ m). Their mean fluorescence value was 0.142 and their fluorescence distribution was smaller than that of all nonstained faecal suspensions. To determine the specificity of the measurement of '*in vivo*' IgA coating of faecal bacteria, faecal samples were stained with a nonsense FITC-F(ab')₂-anti-mouse 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 4). However, in one sample a separate strongly IgA coated bacterial population was seen (fig 5). In order to estimate the percentage of faecal bacteria coated with IgA and their levels of fluorescence we performed match region subtraction (26) by 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) adjusted and

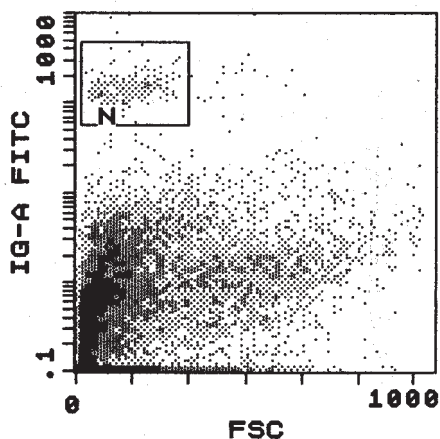


Figure 5. FSC versus FITC-fluorescence distribution of a faecal bacterial suspension stained for IgA. This sample contained a separate strongly IgA coated population which was sorted by flow cytometry (gate N).

thereafter both are subtracted (fig 4). 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 with 1300 MESF. The intra-assay 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 4). 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 faecal samples were analysed by image analysis as well. Since total numbers of bacteria recorded by 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 with 5×10^2 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 with 4×10^2 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 ($\rho = 0.54$, $P < 0.05$, fig 6). In contrast with the Q3, the median specific fluorescence (image analysis) did not correlate with flow cytometry data.

Strongly fluorescent bacteria within a mixed population like faecal flora should be recognised by both systems. Fortunately, one faecal sample contained a separate small population (2%) of strongly FITC-anti-IgA labeled bacteria with a mean FSC of 180 (fig 5). Morphology of these bacteria was determined by image analysis as well as by flow cytometry sorting. The most fluorescent bacteria recorded by image analysis were coccoid rods with a median surface area of $1.3 \mu\text{m}^2$. Flow cytometry sorted bacteria were visually evaluated and were morphologically identical to the coccoid rods recorded by image analysis.

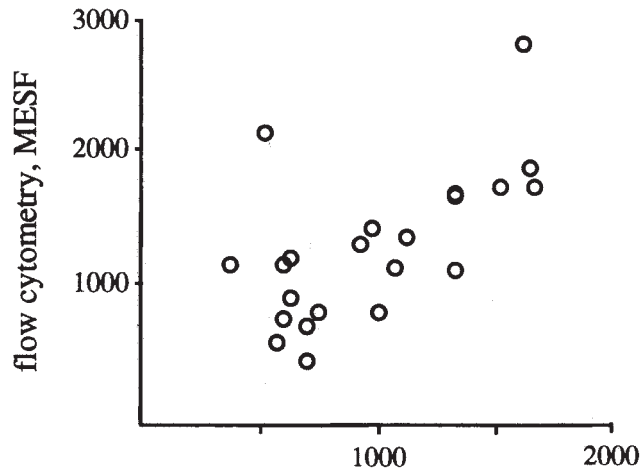


Figure 6. Relation between fluorescence values of bacteria labeled with FITC-anti-IgA of 22 human faecal samples as determined by both methods. MESF values corresponding with the third quartile of specific fluorescence (image analysis) and the mean fluorescence of stained bacteria as determined with match region subtraction (flow cytometry) are plotted.

Discussion

Flow cytometry analysis of noncultured faecal anaerobic bacteria.

In the present study we describe a new rapid flow cytometry based method to analyse noncultured anaerobic bacteria present in human faecal suspensions. Major problems in our approach appeared to be the small size of some faecal bacteria, the presence of non-bacterial objects and bacterial fragments as well as the presence larger objects formed by bacterial aggregates. Since aerobic bacteria comprise less than 0.1 % of the faecal flora, their contribution to the data will be negligible¹⁶.

A discriminator set on FSC, as is commonly used for eukaryotic analysis, is sufficient to analyse suspensions of larger bacteria without interference of background noise^{1,2,4,27}. Our faecal bacterial suspensions, however, appeared to contain small bacteria as well, with a FSC at a level at which background noise is present. As was described by others for aerobic bacteria^{7,28}, we show here that with a trigger set on propidium iodide (PI) staining, small intact anaerobic bacteria can be selectively analysed without interference of background noise. In contrast to aerobic bacteria which need some kind of fixation to become killed and permeable for the fluorescent dsDNA / RNA stain PI, anaerobic faecal bacteria stain with PI without other treatment than exposure to oxygen during sample preparation. The absence of PI⁻ objects with bacterial morphology supports this statement.

A second advantage of a discriminator set on PI-fluorescence is exclusion of PI⁻ non-bacterial objects. A characteristic of most small non-bacterial faecal compounds (e.g. cellular debris,

mucus) and bacterial fragments is that they do not contain dsDNA or RNA and therefore will not stain with PI. Our results show that the discarded PI- events were indeed non-bacterial compounds and bacterial fragments.

Faecal 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 < 1000 all single bacteria were analysed. However, 8% of all particles with a FSC < 1000 were agglutinated small bacteria.

In conclusion, with a discriminator set on PI-fluorescence and exclusion of events with high FSC, most if not all analysed events are intact predominantly single faecal anaerobic bacteria.

Size of anaerobic faecal bacteria.

To our knowledge, no data on the size of bacteria present in faecal suspensions have been published yet. Therefore, faecal samples of 22 healthy volunteers were analysed by flow cytometry and data were validated by analysis of the same samples by computer image analysis as well. Faecal flora consists of a heterogenous population of possibly as many as 400 different species of anaerobic bacteria¹⁵. In faecal suspensions each species is present in low concentrations of < 3 %¹⁴. This heterogeneity was reflected in the large coefficient of variation (CV) of the FSC distributions within a sample, compared to pure cultures. The majority of the analysed faecal bacteria were small, with a FSC corresponding with a 2D-surface area < 1.5 μm^2 . This surface area is smaller than that of pure cultures studied 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 faecal flora composition for which some evidence is found in the literature^{14,16}. In conclusion, faecal suspensions contain a heterogenous bacterial population with large variation in bacterial size. However, most faecal bacteria are small with a 2D-surface area < 1.5 μm^2 .

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 measured FITC fluorescence of 'nonfluorescent' latexparticles. Herewith, the lower detection limit of logarithmically recorded fluorescence of our flow cytometer was found to be as low as 3×10^2 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 faecal anaerobic bacteria.**

One of the aims of this study was to determine the technical possibility of measurement of *in vivo* IgA coating of anaerobic bacteria present in faecal suspensions by flow cytometry. In the gut lumen, IgA is secreted by the intestinal mucosa in large amounts and specificity for intestinal anaerobic bacteria is present¹⁰. All faecal samples were found to contain IgA coated as well as non-IgA coated bacteria. The mean fluorescence of non-IgA coated bacteria was 2×10^2 MESF and presumably composed of photomultiplier background noise and autofluorescence.

In most samples there was not a 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 fitting²⁶. The median percentage of IgA coated bacteria was 45 % with a mean fluorescence of 1300 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, by for instance 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 autofluorescence to image analysis data due to less selective fluorescence filters. These increased autofluorescence values resulted in a decreased relative difference between the median fluorescence of nonstained bacteria (autofluorescence) 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 by ELITE software is presumably more approximate 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 faecal bacteria with high sensitivity.

Comparison of flow cytometry with image analysis

In order to compare flow cytometry with our image analysis system^{21,22}, analogous parameters of bacterial size and fluorescence were measured of pure cultures and all 22 human faecal samples. In contrast 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 only be partially eliminated from further evaluation by morphological criteria. We found a linear relation between FSC and bacterial 2D-surface area as was determined with analysis of several pure cultures by flow cytometry as well as image analysis. Robertson et. al. however, found a linear relation between FSC and bacterial volume⁶. 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 with 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 ($0.15 \mu\text{m}^2$) than for flow cytometry ($0.5 \mu\text{m}^2$). However, flow cytometry acquisition rate is 1,000 x higher compared to image analysis. Flow cytometry, furthermore, was more effective in the reduction of autofluorescence, 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 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 by 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.

Conclusion

In conclusion, with flow cytometry it is possible to analyse intact single faecal bacteria within highly heterogeneous bacterial populations and without interference of contaminating non-bacterial compounds, bacterial fragments and aggregates. Furthermore, it is possible to measure low levels of bacterial *in vivo* IgA coating by means of immuno-fluorescence. 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 by both methods were correlated. This validates both methods.

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