Circulating antibodies against faecal bacteria assessed by immunomorphometry: combining quantitative immunofluorescence and image analysis

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SUMMARY

A new technique to study the prevalence of circulating antibodies directed against different morphological groups ('morphotypes') of bacteria in fresh faeces is presented. The technique combines quantitative indirect immunofluorescence with digital image analysis. Plasma antibody titres and patterns of IgA, IgG and IgM isotype against morphotypes of faecal bacteria were determined in ten healthy individuals.

INTRODUCTION

Although the intestinal indigenous microflora is generally assumed to live peacefully in symbiosis with the host [1], there is increasing evidence that the composition of the indigenous intestinal microflora might play an important role in several diseases, such as autoimmune disorders [2, 3] and chronic graft-versus-host disease [4]. The pathogenesis of these diseases could be antibody mediated. Therefore, we decided to develop a technique to determine the interaction between the microflora and the immune system, in a qualitative and quantitative way.

Culturing, especially of obligate anaerobic bacteria for determining antibodies to individual strains of species, is very laborious. Moreover, culturing may alter the antigenic properties of the bacterial cell wall, as demonstrated in facultatively anaerobic Gram-negative bacilli [5]. A direct method for the assessment of antibacterial antibodies without the need to culture any strain was sought.

Some investigators managed to divide intestinal bacteria into morphologically defined groups by manual assignment [6, 7]. However, in our laboratory we have been less successful in manually assigning bacteria. Therefore, a computerized image analysis system was developed which enables objective classification of each bacterium according to morphological parameters [8]. We have previously shown that in case of pure cultures of Enterobacteriaceae, it is possible to assess serum antibodies by indirect immunofluorescence. This was achieved by measuring the immunofluorescence per bacterium in a quantitative way by computerized image analysis [9]. By combining the indirect immunofluorescence method and the computerized image processing, one has a tool for the assessment of the (plasma) antibody response of several isotypes against morphologically defined groups of

bacteria ('morphotypes'), in an objective way. Therefore, instead of measuring antibodies against pure cultures, antibodies were measured against all bacteria present in faeces in concentrations of 10^8 c.f.u./g and more.

Our first step in this research, however, was to find out whether antibacterial antibodies against the indigenous faecal microflora actually exist. In the present study the method for assessing the antibody response against different morphotypes of intestinal bacteria is described. Plasma was used instead of serum for measurement of the antibodies because the method is designed amongst others for a future study in patients with systemic lupus erythematosus (SLE). In these patients plasma samples are preferred rather than serum samples, because of the fact that many anti-DNA antibodies are lost in serum samples and not in plasma samples. Plasma antibody titres and patterns were determined in ten healthy individuals.

MATERIALS AND METHODS

Volunteers

Ten healthy volunteers, three males and seven females, aged 22–58 years, provided faecal and blood samples after they had given informed consent.

Plasma

Blood samples, collected in glass tubes containing EDTA, were centrifuged in a Beckman centrifuge type TJ-6 (Palo, Alto, California, USA) for 10 min, at 1420 g. The plasma was stored at -20 °C in aliquots of 0·2 ml.

Faeces

The fresh faecal samples were stored at -20 °C until use.

Indirect immunofluorescence (IIF) of faecal samples

Faecal samples were thawed and 0·5 g per sample was suspended in 4·5 ml demineralized water with 0·5 % Tween 80 (Merck, Darmstadt, FRG) and some glass beads. The suspension was centrifuged in a Beckman centrifuge type TJ-6, for 10 min, at 7 g, to separate the bacteria from the larger non-bacterial particles. To reduce spontaneous agglutination of the bacteria on the slide the 0·5 % Tween 80 in demineralized water was added (1:250, v/v, final dilution) to the supernatant. This concentration has been found to have no influence on the titres. Ten microlitres of the bacterial suspension was placed in each well of a series of 12-well IIF slides (Immunocor, Limoges, France). These slides had been degreased in ethanol 96 % and then in acetone. The slides were allowed to dry on a 'handwarm' hotplate. Fixation was performed for 10 min in acetone. The slides were washed gently in phosphate-buffered saline (PBS, pH = 7·2) for 5 min, and dried at room temperature in a cool-air current from an electric fan. In the meantime serial twofold plasma dilutions in PBS were prepared ranging from 1:8 to 1:64. Twenty microlitres of each plasma dilution was then added to the wells on the slide; instead of plasma, PBS was added to the fifth well as a control. After incubation for 45 min in a moist chamber at 21 °C the slides were gently washed three times for 5 min in PBS of 37 °C. The slides were dried and 20 ml fluorescein isothiocyanate (FITC)-conjugated goat anti-human F(ab')₂ IgA, IgG and IgM (Kallestad, Texas, USA) (1:100, v/v, in PBS containing 0·5 % BSA) was added to
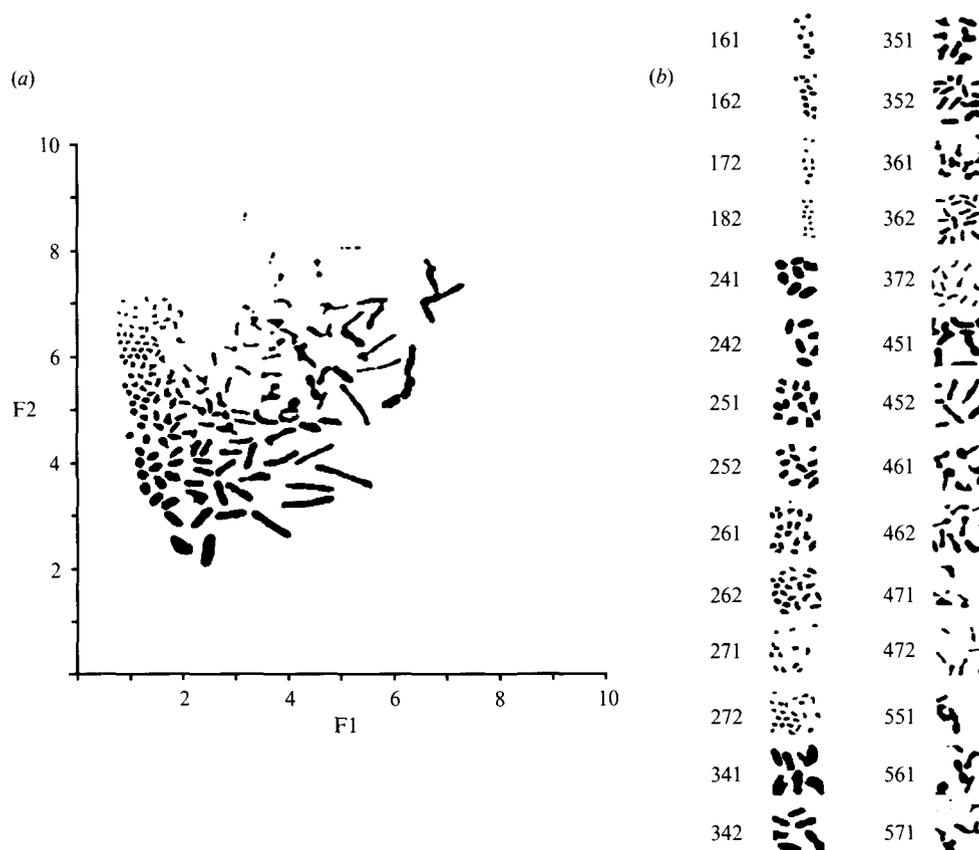
Fig. 1(*a*) Faecal objects (mainly bacteria) of a healthy volunteer are depicted according to their scores of the morphological parameters F1 and F2. The axes were divided in ten equal parts, according to which the morphotypes were defined. (*b*) Representative forms of objects belonging to the 28 morphotypes.

each well. After incubation for 60 min in a moist chamber at 21 °C the slides were again gently washed three times for 5 min in PBS of 37 °C. Mounting fluid, consisting of glycerol/Tris HCl, pH 8·7, (1:1, v/v) was then added to the slides. Finally the slides were covered with a cover slide, which was fixed with nail polish at the sides and stored in dark at 4 °C until examination within 24 h. The plasma antibodies of the IgA, IgG and IgM isotype were determined in duplicate for every faecal sample.

Reading of the immunofluorescence slides

We used a microscope (Olympus BH2, Olympus Optical Co. (Europa) GMBH, Hamburg, Germany) equipped with a phase-contrast condensor and halogen lamp (HBO 50 W, Osram , Berlin, Germany). A videocamera (Fairchild CCD 50001/1, Fairchild Weston Systems Inc., Sunnyvale, California, USA) was placed on top of the microscope. The camera was connected to an 80386-based AT compatible computer with a Matrox MVP-AT(N/P) image processor board and monitor. Images recorded by the videocamera were displayed on a high-resolution colour screen.
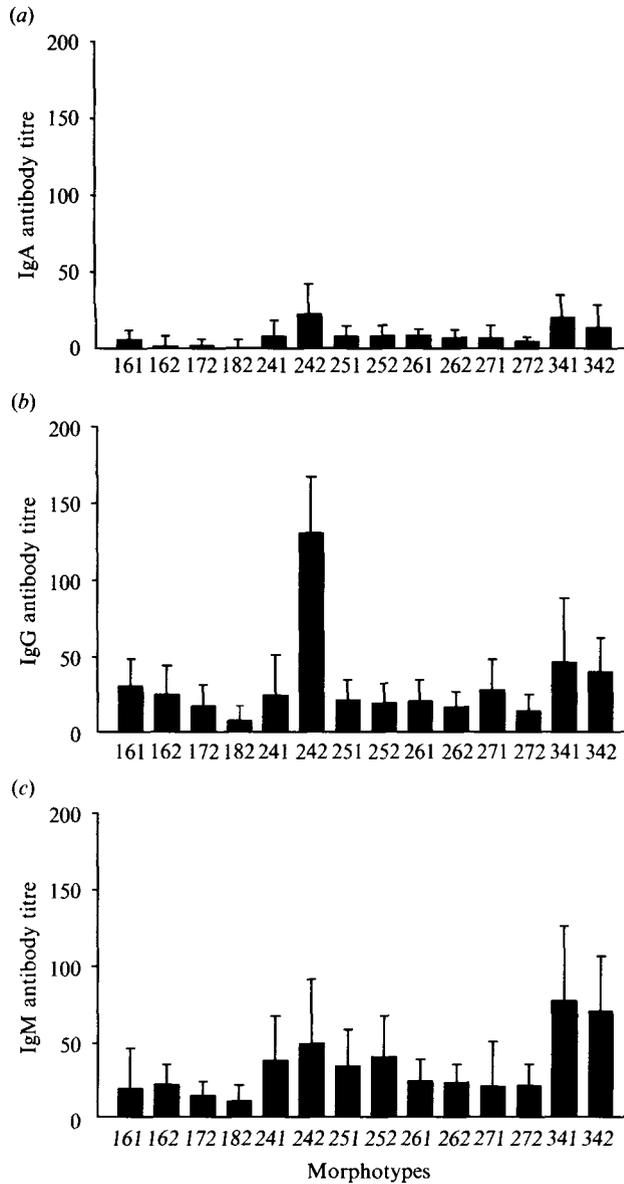
Fig. 2 For legend see opposite.

The image acquisition software was developed specifically for this application in our laboratory. The analysis package used was an adaptation of the morphological package developed by Meijer and co workers [8]. Apart from the fluorescence data, morphological information was computed for every object in the field of view.

Determination of the antibody titres for each morphologically defined group of bacteria (morphotype) with the image processing system

For each plasma dilution the fluorescence intensity of each bacterium was determined. Per plasma dilution about 1000 objects (predominantly bacteria)
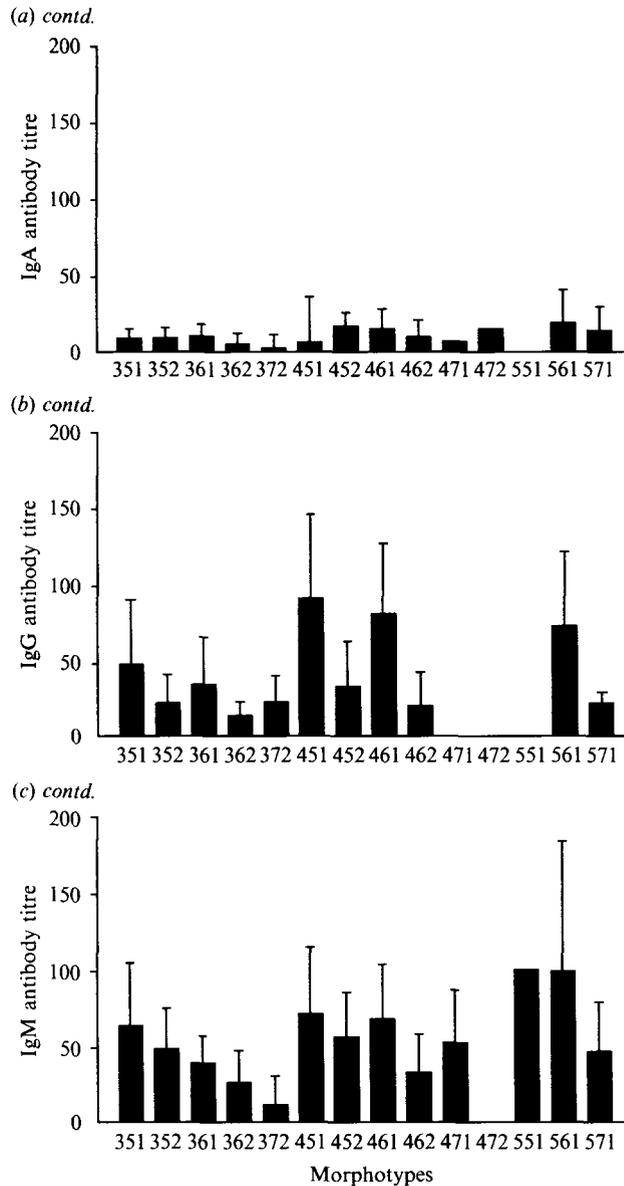
(*a*) *contd.*

(*b*) *contd.*

(*c*) *contd.*

Morphotypes

Fig. 2(*a*) Mean (±s.d.) plasma antibody titres of the IgA isotype against objects composing the faecal flora of ten healthy individuals. (*b*) Mean (±s.d.) plasma antibody titres of the IgG isotype against objects composing the faecal flora of ten healthy individuals. (*c*) Mean (±s.d.) plasma antibody titres of the IgM isotype against objects composing the faecal flora of ten healthy individuals.

were measured. The objects got assigned three morphological parameters: F1, circular versus oblong forms, F2, size of the projected area, and F3, irregularity. Faecal objects (predominantly bacteria) of a healthy volunteer are depicted in Fig. 1 (*a*) according to their morphological parameters F1 and F2. From Fig. 1 (*a*) it is clear that the objects can be classified into morphotypes on the basis of their

morphological characteristics [8]. Therefore, the scales for the morphological parameters F1 and F2, were divided into ten equal parts forming a grid, because these parameters explain most of the morphological variation of the sample [8], the scale for parameter F3 was divided into only two equal parts, resulting in the morphotypes shown in Fig. 1 (*b*).

Therefore, for each morphotype the median fluorescence intensity of the objects (mainly bacteria) belonging to that morphotype was determined. The median was calculated only if the morphotype contained a minimum of ten objects. For each morphotype, linear regression was performed on the median fluorescence intensity as a function of the reciprocal plasma dilution, again only if the morphotype contained at least ten objects in every plasma dilution. The slope and intercept of the resulting linear fit were interpreted as indicative of antibody concentration and background brightness level, respectively. To calibrate the slope, a threshold at which the human eye observes positive fluorescence, was determined as described previously [9].

Sensitivity

In order to determine the sensitivity of the method, we mixed a known *Enterobacter intermedius* strain with faeces of a volunteer in the proportions 124:1, 14:1, 1·4:1, 1:7·25. Thereafter, we determined plasma IgG antibody titres against these mixtures and against faeces along as described above. The plasma as well as the *E. intermedius* strain, were obtained from a patient with an *E. intermedius* septicaemia. The plasma antibody titre against the *E. intermedius* strain was 1024. The titre against this *E. intermedius* strain was high compared to the titres against the indigenous faecal bacteria. In this way we measured how many *E. intermedius* bacteria had to be minimally present in a faecal sample in order to be detected by this method. The concentration of the *E. intermedius* strain, grown overnight in Brain Heart Infusion broth (BHI, Oxoid, Basingstoke, UK), was assessed with a poured plate dilution technique. The concentration of the faecal bacteria was measured by direct microscopic clump counts (DMCC) [10].

Statistics

For each isotype the absolute difference between duplicate measurements per morphotype was taken as a standard deviation of the measurement, as an indication of the accuracy of the method.

RESULTS

Plasma antibody titres for the isotypes IgA, IgG and IgM are depicted in Fig. 2. The inter-individual range in titre can be read from the figure. Plasma antibody titres of the IgA class were low compared to the plasma antibody titres of the IgG and the IgM class. Therefore, we investigated whether this could be due to *in vivo* coating of the bacteria with IgA. We measured positive fluorescence of objects in the PBS controls of all samples for the three antibody isotypes. Indeed 7·3% of the objects in the PBS control were IgA coated, 6·6% with IgG and 6·2% with IgM. This means that there was no significant difference in percentage of *in vivo* coated bacteria between the three isotypes.
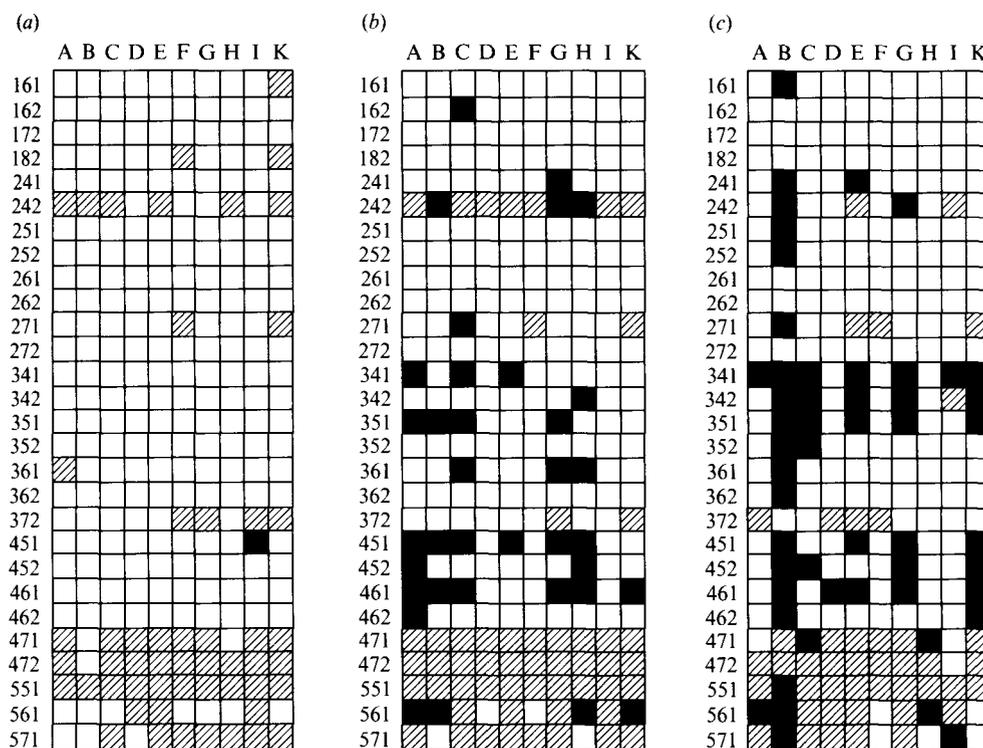
Fig. 3 (*a*) The IgA plasma antibody responses against his/her own faecal morphotypes are shown for each person (A–K). Titres higher than 64 are represented by black boxes whereas morphotypes with titres lower than 64 are left open (white boxes). When too few bacteria were found in a morphotype to calculate the titre, the box was hatched. (*b*) The IgG plasma antibody responses against his/her own faecal morphotypes are shown for each person (A–K). (*c*) The IgM plasma antibody responses against his/her own faecal morphotypes are shown for each person (A–K).

When two faecal samples are compared, it is important to know per morphotype which differences in titre are significant. The difference between duplicate measurements was considerable compared to the differences between duplicate measurements in pure cultures [9]. For IgA the mean absolute difference (S.D.) between duplicate titre measurements for a morphotype was $3\cdot3$ $\log_2$ titre steps. For IgG the mean absolute difference was $3\cdot7$ $\log_2$ titre steps and for IgM the mean absolute difference was $4\cdot4$ $\log_2$ titre steps.

The individual pattern of plasma antibody titres for each person is shown in Fig. 3. This enabled assessment of intra-individual and inter-individual range. When too few bacteria were present in a certain morphotype to determine the antibody titre against that morphotype, the box representing that morphotype was hatched. There are some inter-individual differences in the kind and the number of hatched boxes representing morphotypes.

The results presented in Fig. 3 show that different immune responses were mounted against all morphotypes. Some morphotypes seemed to be more immunogenic than others. Most individuals had IgG antibodies against the morphotypes encoded 451 (6/10), 461 (6/10), 562 (4/10), and 351 (4/10), while

most persons had IgM antibodies against the morphotypes encoded 341 (7/10), 461 (5/10), 351 (5/10), 3423 (5/10), 451 (4/10), and 452 (4/10).

Regarding the sensitivity it appeared that in the proportion of 14:1 and higher the fluorescing antibody coated *E. intermedius* could be easily be detected. The pure culture of *E. intermedius* was not confined to just one morphotype, but ranged over several adjacent morphotypes: 241, 242, 341, 342, 451, 551.

DISCUSSION

In the present study it was found to be possible to determine titres of circulating antibodies and their isotypes against bacteria in faceal samples which are present in concentrations of 10^8 c.f.u./g and higher. When our method low antibody titres were found against many morphologically different faecal bacteria in healthy human individuals.

There are inter-individual differences in the kind and the number of morphotypes that contain too few objects to enable calculation of the antibody titre against that particular morphotype. This is due to the fact there there are inter-individual differences in the micromorphology of faecal bacteria between healthy volunteers as was shown by Meijer [11].

Per individual, the pattern of the plasma antibodies against the various morphotypes differed significantly and considerably. Interestingly, an analogous diversity of (natural) antibody patterns has been described directed against both tissue specific and not issue specific antigens in healthy individual as well as in patients with SLE [12]. In case of tissue antigens, shared by all humans, variation in antibody response directed against such antigens could be ascribed to variation in immune reactivity between individuals. In case of faecal bacterial antigens, inter-individual variation could firstly be due to the fact that not every person encounters the very same bacterial antigens. If a bacterium with its antigen has never entered the digestive tract, no antibodies can have been produced. Secondly, the variation between no response and a titre higher than 64 in antibody responses between individual could be due to variation in immune reactivity. The latter is of particular interest, since the individual immune response towards certain bacterial antigens might influence the course of autoimmune disease in certain patients, e.g. with SLE.

In vivo, 7·3% of the bacteria under study were coated with IgA (PBS controls). Surprisingly the amount of *in vivo* coating with IgG and IgM was comparable to the percentage of IgA coating. Usually the local IgA production in the digestive tract exceeds the local production of IgM and IgG. Obviously, this large amount of local IgA is not directed towards the intestinal bacteria under study, representing the populations present in concentrations of 10^8/g and higher. These *in vivo* data contrast with the systemically circulating antibodies: here IgA titres were found to be very low in comparison with IgG and IgM titres (Fig. 2a, 2b and 2c). A varying percentage of the intestinal bacteria concentrated 10^8/g and higher had apparently been able to evoke a systemical humoral immune response.

An indication of the sensitivity of the method was given by the test with a *E. intermedius* strain, against which a high antibody titre existed, that was mixed in several proportions with faeces. In a mixed population of 100 bacteria, there must

be minimally seven bacteria of a particular strain with about the same morphological characteristics, to be detectable amongst the others with sufficient significance.

The faecal microflora can be regarded as a very complex system. Therefore, it is obvious that our classification of the faecal microflora into 28 morphotypes has no relation with conventional bacteriology. The morphotypes do no refer to bacterial genera or even families. This was illustrated by the fact that a pure culture of *E. intermedius* was not confined to just one morphotype, but ranged over several adjacent morphotypes as was also found by Meijer [8]. The reverse is also true, e.g. two bacteria belonging to the same morphotype do not necessarily belong to the same species. Therefore, the results cannot be interpreted in terms of bacterial species, but rather indicate humoral reactivity towards the more abstract populations of morphologically similar objects like morphotypes. These facts also imply that for faecal flora the standard deviation in the assessment of the titre per morphotype could not be as small as in the case of pure cultures [9].

We have developed this direct 'immunomorphometric' test, since it reflects the actual *in vivo* situation of the (serological) composition of the intestinal flora as closely as possible. Considering the fact that this kind of information could not be obtained otherwise, the method may became a valuable tool for study of host-microflora interactions. For example, studies of the interaction of the microflora an the immune system may become clinically relevant when we focus on the pathogenesis and the maintenance of autoimmune diseases such as SLE. A study of this kind of SLE-patients is currently being undertaken.

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