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# Host-microflora interaction in systemic lupus erythematosus (SLE): circulating antibodies to the indigenous bacteria of the intestinal tract

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## SUMMARY

Experimental data suggest a role for the microflora in the disease expression of systemic lupus erythematosus (SLE). In active SLE anti-ds-DNA antibodies are supposed to be pathogenic by forming immune complexes with DNA. Bacteria might induce the production of anti-ds-DNA antibodies. To explore the relation between the host and his microflora in SLE in comparison with healthy controls we studied the prevalence of systemic antibodies to faecal bacteria that were discriminated by their morphology by indirect immunofluorescence.

IgM titres against their own faecal microflora were found to be lower both in active and inactive SLE when compared to healthy individuals. IgG-class antibacterial antibodies were increased in inactive SLE but decreased in active SLE compared to inactive SLE and healthy controls, although plasma levels of total IgG were almost doubled in active SLE. The lower IgG antibacterial antibody titres in active SLE might possibly result from sequestration of these IgG antibodies in immune complexes, indicating a possible role for antibacterial antibodies in exacerbations of SLE.

## INTRODUCTION

Systemic lupus erythematosus (SLE) is a disease of unknown etiology characterized by hypergammaglobulinaemia and the production of multiple autoantibodies. Amongst these, anti-ds-DNA antibodies are both sensitive and specific for the disease [1]. Rises in anti-ds-DNA antibody levels were shown to parallel or precede clinical parameters of disease activity [2]. These and other data suggest that anti-ds-DNA antibodies play a role in the pathogenesis of exacerbations of SLE supposedly by forming immune complexes with DNA. The stimulus for the *in vivo* anti-ds-DNA antibody production in SLE, however, is unknown.

There is increasing evidence that in autoimmune-prone mice the composition of the indigenous intestinal microflora plays an important role in the expression of autoimmune disorders [3–5]. Bacterial products such as lipopolysaccharides and

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peptidoglycans may cause polyclonal B cell activation resulting in the production of antibodies with a repertoire similar to that of humans with systemic autoimmune disease [6]. Polyclonal B cell activation has been shown to precede the development of autoimmune phenomena in lupus-prone mice [7]. Besides autoantibody production as a result of polyclonal B cell activation, there is evidence that disease activity in SLE is accompanied by antigen-driven production of autoantibodies [7, 8].

Bacteria can not only deliver polyclonal activators for B cells but may also induce antigen-driven production of anti-ds-DNA antibodies on the basis of cross-reactivity between bacterial and human DNA [9]. It has been shown that patients with SLE have higher levels of antibodies against DNA of a wider variety of bacterial species than healthy individuals [10]. In addition, anti-DNA antibodies can be induced in mice by immunization with bacterial DNA. These anti-DNA antibodies resemble some anti-DNA antibodies expressed in spontaneous autoimmune disease in these mice [11]. Thus, bacteria can provide both polyclonal activators and cross-reacting antigens for the production of anti-DNA antibodies and thereby may play a role in the pathogenesis of systemic autoimmune disease [12, 13]. The bacteria involved might originate from the intestinal tract, since it was found that the DNA component of circulating immune complexes of patients with SLE hybridized with the lacZ gene of *Escherichia coli* [14]. Furthermore, NZB/NZW mice on milk of cows immunized against 26 strains of intestinal bacteria had lower titres of anti-ss-DNA antibodies and a delay in the occurrence of nephritis [15].

We studied the antibody repertoire against indigenous intestinal bacterial antigens in SLE patients with active and inactive disease in comparison with healthy controls. We hypothesized that patients with SLE have higher levels of antibodies against a wider range of indigenous bacterial antigens than healthy individuals as this quantitative and/or qualitative difference in antibody repertoire might underlie the occurrence of autoimmune phenomena. The humoral immune response against the indigenous intestinal microflora was evaluated by means of a direct immunomorphometric test. This test enables the measurement of IgA-, IgG- and IgM-isotype titres against intestinal bacteria present in faeces without the need to culture the bacteria [16].

## SUBJECTS AND METHODS

*Patients and healthy individuals.* Twenty-four patients (19 women and 5 men), median age 32 years (range 17–66) and 19 healthy volunteers from the same hospital environment (16 women and 3 men), median age 32 years (range 22–58), entered this study after having given written informed consent. The patients and the healthy volunteers had not received prednisone in a dosage of more than 30 mg daily in a period of 3 months preceding the study period nor had they had any medication influencing gastrointestinal motility or permeability. High doses of prednisone were excluded because they are known to influence the production of antibodies. Other medication that was administered was: none in the healthy control group ($n = 19$); in the inactive SLE group ($n = 16$): daily anti-malarials ($n = 8$), daily azathioprine ($n = 2$), daily low dose prednisone ($n = 8$) and

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cyclophosphamide 750 mg every 3 months ($n = 1$). In the active SLE group ($n = 8$): daily anti-malarials ($n = 3$), daily azathioprine ($n = 1$), daily low dose prednisone ($n = 1$). This medication is not known to influence the intestinal microflora and, except for azathioprine and cyclophosphamide, hardly influences the production of antibodies, such as the antibodies against the faecal microflora in our study. In the period of 1 month before and during the sampling no one had suffered from a gastrointestinal disease or had taken antibiotics.

The study was approved of by the Medical Ethics Committee of the University Hospital Groningen.

*Plasma.* A blood sample was taken from each individual, collected in glass tubes containing EDTA and centrifuged in a Beckman centrifuge type TJ-6 (Palo Alto, Calif., USA) for 10 min, at 1420 g. The plasma was stored at −20 °C in aliquots of 0·2 ml.

*Faeces.* A fresh faecal sample was obtained from each subject and was stored at −20 °C until use.

*Assessment of disease activity.* Active disease was defined as the occurrence of a minor or major disease exacerbation at the time of sampling according to criteria described previously [2]. If the patient did not fulfil those criteria the disease was considered inactive. To assess disease activity Liang's SLAM index [17] was also calculated at the outpatient clinic visit at the end of the 2 weeks sampling period.

*Indirect Immunofluorescence (IIF) of faecal samples*

*Immunomorphometry.* We performed an indirect immunofluorescence test to measure the antibacterial antibodies. The slides were read by an image analysis system, which divided the bacteria into different groups (morphotypes) on the basis of their micromorphology (Fig. 1) and measured the antibody titres of the IgA-, IgG- and IgM-isotype against these morphotypes as described previously [16]. In brief, faecal samples were thawed and 0·5 g per sample was suspended by vortexing in 4·5 ml demineralized water with 0·5% Tween 80 (Merck, Darmstadt, FRG) and some glass beads. The suspension was centrifuged slowly at 7 g for 10 min, to separate the bacteria from the larger non-bacterial particles. The supernatant was diluted in demineralized water with 0·5% Tween 80 to a final dilution of 1:250 (v/v). Ten microlitres of the bacterial suspension were 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 and 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. Serial twofold dilutions of plasma in PBS were prepared ranging from 1:8 to 1:64. Twenty microlitres of each plasma dilution and one PBS control were then added to the wells on the slide. After incubation for 45 min in a moist chamber at 21 °C the slides were gently washed again. The slides were dried and 20 $\mu$l of fluorescein isothiocyanate (FITC)-conjugated goat anti-human $F(ab')_2$ IgA, IgG and IgM (Kallestad, Tex., USA) (1:100, v/v, in PBS containing 0·5% BSA) were added to the wells. After incubation for 60 min in a moist chamber at 21 °C the slides were again gently washed. 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

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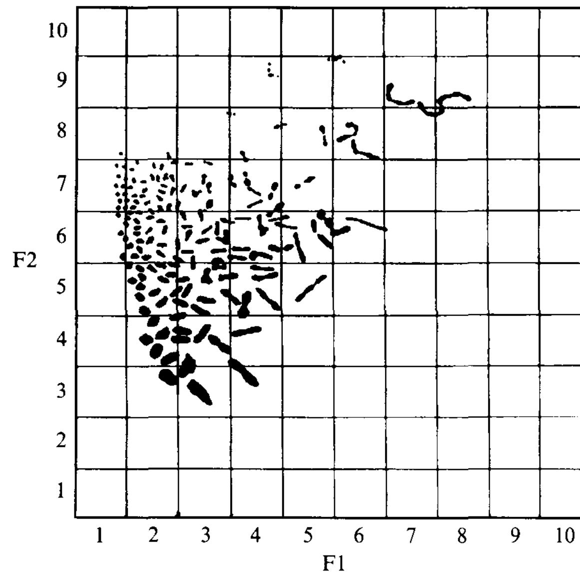


Fig. 1. Faecal objects (mainly bacteria) of a healthy volunteer are depicted according to their morphological parameters F1, distinguishing circular versus oblong forms, and F2, representing the size of the bacteria. The third morphological parameter, F3, for irregularity, is not shown. The axes, representing F1 and F2, were divided in 10 equal parts and F3 was divided in only two equal parts, according to which the morphotypes were defined.

slip, that was fixed with nail polish at the sides, and stored in the dark at 4 °C until examined within 24 h. All determinations were done in duplicate for every faecal sample.

*Total plasma IgA, IgG and IgM levels.* Total plasma IgA, IgG and IgM levels were measured by nephelometry.

*Statistical analysis.* Levels of total plasma IgA, IgG and IgM were compared between patients with inactive and active SLE and healthy controls using Wilcoxon's test. The distribution of morphotypes (micromorphologically defined groups of faecal bacteria) of the faecal flora of healthy individuals and patients with inactive and active SLE were compared by univariate F tests. Differences in plasma antibody titres against the morphotypes of patients with active and inactive SLE and healthy individuals were assessed by the ANOVA F-test applied to logarithmically transformed titres. Tests were performed at the significant level of 0·05.

## RESULTS

### *Healthy individuals*

The antibody responses to indigenous intestinal (faecal) bacteria for the isotypes IgA, IgG and IgM are depicted in Figures 2*a–c*. The results in Figures 2*a–c* show that in healthy individuals different immune responses were mounted against their intestinal bacteria. The antibodies were not directed against selected morphologically defined groups (morphotypes) of faecal bacteria, but against all faecal morphotypes. IgA antibody titres were much lower than antibacterial antibody titres of the IgG- or IgM-isotype.

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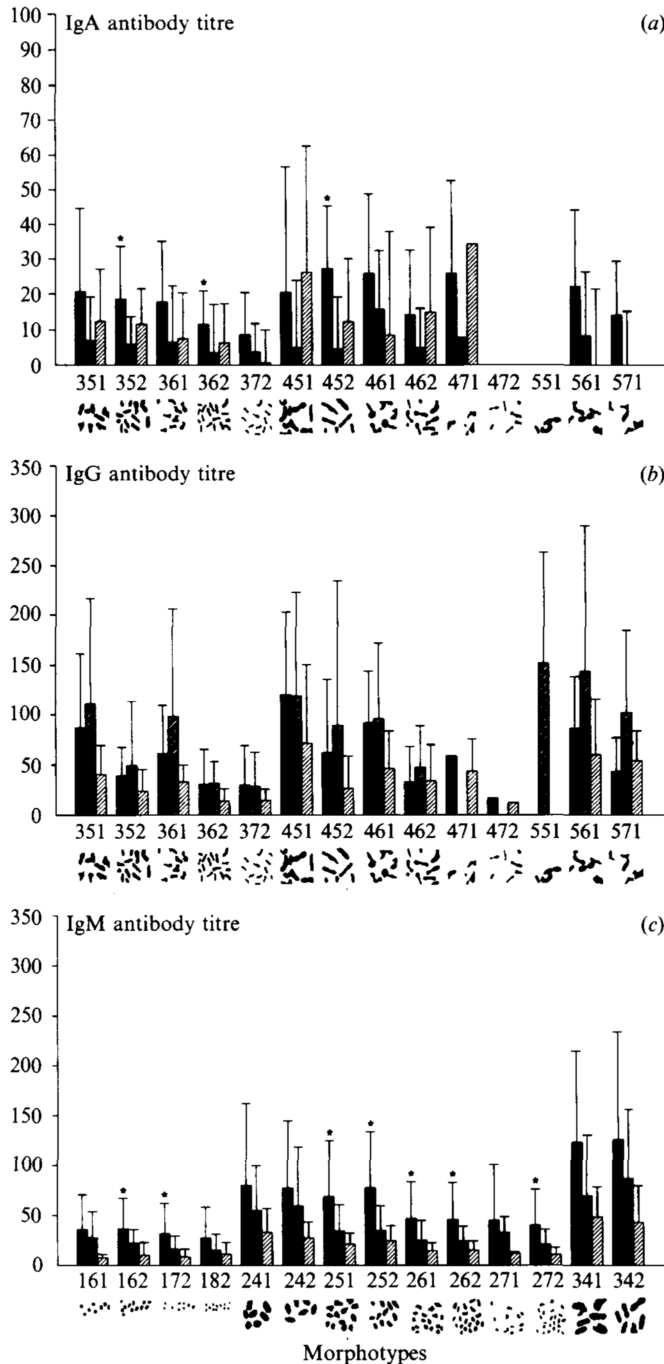


Fig. 2. (*a*) Mean (+S.D.) plasma antibody titres of the IgA-isotype against morphotypes of the faecal flora of healthy individuals ($n = 19$), patients with inactive SLE($n = 16$) and patients with active SLE ($n = 8$). On the $x$-axis the different morphotypes are depicted with their respective code, on the $y$-axis the IgA antibody titre is given [16]. Significant differences in titres between the three groups are marked with an asterisk. (*b*) Idem for IgG. (*c*) Idem for IgM. The first closed bar denotes healthy individuals, the second hatched bar denotes patients with inactive SLE, and the third bar denotes patients with active SLE.

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*Patients with inactive SLE*

There were no differences in the distribution of the morphotypes in SLE patients and healthy individuals. The number of bacteria within a certain morphotype was comparable in every person. Some morphotypes seemed to be more immunogenic than others. For IgA circulating antibody titres against various morphotypes were lower in patients with inactive SLE than in healthy persons (Fig. 2*a*). Significant differences were found for the morphotypes 262, 352, 362, and 452. On average IgG plasma antibody titres were higher in inactive SLE than in normal controls (Fig. 2*b*). Differences were significant for the morphotypes 271 and 341. IgM antibody titres were lower in active SLE compared to healthy individuals (Fig. 2*c*). Results differed significantly for the morphotypes 162, 172, 251, 252, 261, 262, 272, 352, 361, 452 and 461.

*Patients with active SLE*

The mean (S.D.) SLE activity score according to Liang and colleagues in the patients assigned to the group with inactive SLE was 5·2 (1·76), the mean (S.D.) SLE activity score in patients assigned to the group with active disease was 14·4 (2·64). In active SLE patients plasma antibody titres of the IgA class also were low compared to titres of the IgG and the IgM class. For IgA circulating antibody titres against different morphotypes were lower in active SLE than in inactive SLE for the morphotypes 241, 242, 271 and 372. For the other morphotypes IgA titres were higher in active SLE than inactive SLE. For the morphotypes 182, 451 and 471 the IgA titres were even higher than in healthy controls. Differences between the three groups were significant for the morphotypes 262, 352, 362 and 452 (Fig. 2*a*). Surprisingly, the lowest IgG titres were found in the active SLE group (Fig. 2*b*). Differences were significant for the morphotypes 271, 272 and 341. For IgM the titres were also lowest in the active SLE group. Results differed significantly for the morphotypes 162, 172, 251, 252, 261, 262, 272, 352, 361, 452 and 461 (Fig. 2*c*).

Mean (S.D.) total plasma IgA levels in the inactive SLE group were 3·2 (1·7) g/l, in the active SLE group 3·3 (1·9) g/l, in healthy controls 2·8 (1·1) g/l. Mean (S.D.) total plasma IgG levels in the inactive SLE group were 13·6 (4·5) g/l and in the active SLE group 24·7 (9·6) g/l, in healthy controls 14·3 (3·1) g/l. Mean (S.D.) total plasma IgM levels in the inactive SLE group were 2·3 (2·6) g/l and in the active SLE group 3·2 (1·9) g/l, in healthy controls 3·6 (1·6) g/l. For IgG the total plasma levels were significantly higher in active SLE than in inactive SLE ($P < 0·005$) and higher than in healthy controls ($P < 0·005$). For IgM the total plasma levels were significantly lower in inactive SLE than in healthy controls ($P = 0·001$).

## DISCUSSION

The results of our study indicate that in healthy individuals circulating antibodies against faecal bacteria exist of the IgA-, IgG- and IgM-isotype. These antibodies are directed against all morphologically defined different groups of bacteria. The antibodies could have been produced as a result of invasion or translocation of the intestinal bacteria across the gut wall. Little is known on translocation of these mainly anaerobic bacteria across the intestinal epithelium.

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Our findings show that there are obvious differences between SLE patients and healthy individuals with respect to their antibody titres against indigenous gastrointestinal bacteria. The morphotypes 262, 352 and 452 show reactivities with significant differences between SLE and healthy controls in both IgA and IgM morphotypes. With respect to the IgA antibody titres, the differences between healthy controls and SLE patients for the morphotypes 262, 352, 362 and 452 are larger than between active and inactive SLE. Compared to the healthy controls this might suggest that local immunity in SLE is worse than in healthy persons provided that systemic IgA titres parallel IgA titres in the gut [18].

In comparison with healthy individuals both antibacterial IgM titres as well as total IgM levels were found to be lower in patients with inactive SLE. This decrease of IgM possibly could be interpreted in terms of a failing network against bacterial antigens [20] with a subsequent rise of specific (IgG) antibody responses, as was found in patients with inactive SLE. Polyclonal activation usually yields IgM antibodies. Since IgM antibodies against bacteria are lower in SLE than in healthy controls, polyclonal activation by bacteria as a cause of high autoantibody levels is not likely. In laboratory animals it was found that polyclonal activation preceded antigen driven autoantibody production of IgG isotype [7]. In the SLE patients polyclonal activation may not be important as a source for IgM autoantibodies, possibly reflecting that these patients are not in a very early stage of their disease. However, we found higher IgG antibacterial antibody titres in the group with inactive SLE in comparison with the group of healthy individuals. The higher IgG antibody titres in inactive SLE might be caused by antigen driven production. In the group with active SLE a much lower level of IgG antibacterial antibody titres was found, although the concentration of total plasma IgG was almost doubled in comparison with the concentrations in the group with inactive SLE and healthy controls. This cannot be explained by a wider use of immunosuppressive drugs in active patients as azathioprine and cyclophosphamide were not used more frequently in active patients. The difference in IgG antibody titres between patients with active and inactive SLE was significant for a small number of morphotypes only. An explanation for the low titres of a selected group of antibacterial IgG antibodies in patients with active SLE might be that during increase of disease activity specific antibodies may be bound in immune complexes. This might result in lower plasma levels of those specific antibodies compared to levels in periods of less disease activity. The same phenomenon, i.e. a sharp decrease in antibody level, has been shown for anti-ds DNA antibodies during exacerbations of SLE [2, 19]. Therefore, the fall in antibacterial IgG antibodies in active SLE might possibly indicate that the antibodies against bacterial cells currently under study might play a role in the pathophysiology of exacerbations of SLE. Further proof for this hypothesis should be obtained by dissociating immune complexes from active disease sera and analysing the specificity of the antibodies involved, in particular with respect to their reactivity to bacterial antigens from the host microflora.

In conclusion, our findings support the hypothesis that there are qualitative and/or quantitative differences between SLE patients and healthy individuals with respect to their antibody repertoire directed against indigenous gastrointestinal bacterial antigens. Longitudinal studies are indicated to confirm

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these findings and to elucidate the underlying mechanisms, thereby possibly providing a new approach for the treatment of SLE.

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