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Mette Hvatum, Lars Kanerud, Roger Hällgren and Per Brandtzaeg

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The gut-joint axis: cross-reactive food antibodies in rheumatoid arthritis

M Hvatum,[†] L Kanerud, R Hällgren, P Brandtzaeg

[†]*Deceased*

Authors' affiliations
M Hvatum, **P Brandtzaeg**, Laboratory for Immunohistochemistry and Immunopathology (LIIPAT), Institute of Pathology, University of Oslo, Rikshospitalet University Hospital, Oslo, Norway
L Kanerud, Department of Rheumatology, Södersjukhuset Stockholm, Sweden
R Hällgren, Department of Internal Medicine, University Hospital, Uppsala, Sweden

Correspondence to:
Professor P Brandtzaeg,
Institute of Pathology,
Rikshospitalet,
N-0027 Oslo, Norway;
per.brandtzaeg@medisin.uio.no

Running title: Intestinal food antibodies in rheumatoid arthritis

Key words: rheumatoid arthritis; intestinal mucosa; food antibodies; inflammation

Abbreviations: RA, rheumatoid arthritis; OR, odds ratio; RF, rheumatoid factor; NSAID, non-steroidal anti-inflammatory drug; SSZ, sulfasalazine; AmAc, ammonium acetate; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; OD, optical density; SIgA, secretory IgA.

Background: Patients with rheumatoid arthritis (RA) often feel an association between food intake and their disease severity.

Methods: To substantiate immunologically a connection between RA and intestinal immunity, we measured IgG, IgA, and IgM antibodies to dietary antigens in serum and jejunal perfusion fluid from 14 RA patients and 20 healthy subjects. The antigens originated from cow's milk (α-lactalbumin, β-lactoglobulin, casein), cereals, hen's egg (ovalbumin), cod fish, and pork meat.

Results: In intestinal fluid of many RA patients, all three Ig classes showed elevated food-specific activities. Except for IgM activity against β-lactoglobulin, all other IgM activities were highly significantly increased unrelated to the level of total IgM. The RA-associated serum IgM antibody responses were relatively much less pronounced. Compared with IgM, the intestinal IgA activities were less consistently elevated, with no significant increase against gliadin and casein. Considerable cross-reactivity of IgM and IgA antibodies was documented by absorption tests. Although intestinal IgG activity to food was quite low, it was nevertheless significantly increased against many antigens in RA patients. Three of the five RA patients treated with sulfasalazine for 16 weeks had initially raised levels of intestinal food antibodies; these became normalized after treatment but clinical improvement was better reflected in a reduced erythrocyte sedimentation rate.

Conclusion: The production of cross-reactive antibodies is strikingly elevated in the gut of many RA patients. Their food-related problems might reflect an adverse additive effect of multiple modest hypersensitivity reactions mediated, for instance, by immune complexes promoting autoimmune reactions in the joints.

Patients with rheumatoid arthritis (RA) often feel that there is an association between food intake and their disease activity, but evidence to support such a connection has been contradictory. [1] Reports are usually based on diet experiments with quite different set-ups, followed by some sort of food challenge. Food hypersensitivity in RA does not reflect IgE-mediated allergy, and most studies have concluded rather negatively concerning a possible pathogenic impact of food in RA. Thus, Panush [2] performed blinded encapsulated challenges, and no more than 5% of the RA patients were deemed to show immunological food sensitivity. Nevertheless, a recent large European epidemiological study suggested, by determining odds ratios (ORs) after adjusting for possible confounders, that there is a significant association between inflammatory polyarthritis and a high intake of red meat (OR, 1.9), meat and meat products combined (OR, 2.3), and total proteins (OR, 2.9). [3]

Only few previous reports have considered that a pathogenic dietary effect on RA could depend on a persistent intake of food; a brief test challenge with a relatively small dose might not precipitate clinical symptoms. Studies considering the quantitative variable have in fact tended to provide a positive conclusion concerning the pathogenic importance of food, at least in a significant fraction (20-40%) of the patients. [4][5]

Attempts to identify food-sensitive RA patients by measuring food-specific antibodies or immune complexes in serum have failed. [6] Our previous investigation likewise concluded that serum antibody measurements seldom predict or confirm food hypersensitivity in RA patients; although elevated IgM activities were seen, there was no convincing association with clinical variables or dietetic benefits. [7] Elevated serum IgA activity to gliadin has been reported in RA patients, but the levels were low compared with IgG and IgM directed against the same antigen in patients as well as controls. [8] Raised IgG activity to gliadin was found in 47% of 93 RA patients, and 41% had concurrently IgA rheumatoid factor (RF) with some association to duodenal villous atrophy. [9] However, a subsequent study was contradictory. [10]

Altogether, serum antibodies do not appear to provide an immunological link between diet and RA. The reason might be that activation of the intestinal immune system is not reliably reflected in serum. Notably, the fact that circulating IgA RF is predominantly polymeric supports a mucosal origin. [1] Moreover, RA patients may have occult small intestinal inflammation and increased mucosal permeability independent of the use of non-steroidal anti-inflammatory drugs (NSAIDs). [12][13] Therefore, we measured antibodies in perfusion fluid from the jejunum of RA patients and healthy controls to investigate directly the mucosal immune response to a variety of food proteins.

MATERIALS AND METHODS

Study subjects

We studied 17 patients with seropositive RA diagnosed according to the criteria of the American College of Rheumatology. [14] Their mean age was 50 years (range, 26-70 years) and the mean duration of disease 10 years (range, 9 months-30 years). Twenty healthy subjects served as controls; their sex distribution was similar to the RA patients but because of ethical constraints, we were unable to age-match the controls although the ranges were highly overlapping (mean age, 29 years; range, 23-39 years). Both RA patients and controls were Caucasians and lived on ordinary food with no restrictions; none complained of gastrointestinal symptoms.

The RA patients suffered from active disease as defined by the presence of at least two of the following three criteria: duration of morning stiffness $\geq$60 minutes, tenderness or swelling, or both, of $\geq$ six joints, and an erythrocyte sedimentation rate (ESR) >30 mm 1st h. None of the patients had received treatment with gold, penicillamine, chloroquine, sulfasalazine (SSZ), corticosteroids, or immunosuppressants for the 3 months prior to study

inclusion. Most of them were treated with NSAIDs, but in all except two this treatment was withdrawn for at least three days before intestinal perfusion was performed.

Five of the patients were reinvestigated after treatment with SSZ for 16 weeks; three of these had not received NSAIDs for at least 1 month. The dosage of SSZ was increased with 0.5 g weekly from 1 g/day up to 2-3 g/day.

The patient protocols as well as the sampling of serum and jejunal perfusion fluid were based on informed consent and approved by the local Ethics Committee. Most of the subjects were derived from a previously reported study of microbial antibodies. [15]

Jejunal perfusion fluid

A segment of the jejunum was perfused as detailed elsewhere by a small diameter tube that was 175 cm long and contained six channels. [16] The original report described the insertion of the tube, gastric drainage, inflation of the balloons, and rinsing of the closed intestinal segment. C-labelled polyethylene glycol was used as a volume marker and phenolsulphonphatalein (phenol red) as a marker of the patency of the proximal balloon. The recovery of the volume marker was on average 86 $\pm$10 (SD)% in RA patients and 89 $\pm$6% in controls. The fluid was collected on ice, centrifuged in cold conditions at 2500 *g*, and frozen in aliquots of 2 ml until analysis. Successful sampling was achieved from 14 RA patients and all controls.

Antigens

Antigen solutions were prepared as described. [17] Briefly, gliadin (Karl Roth, Karlsruhe, Germany) and oatE represent ethanol (70%)-extractable prolamins from wheat and oats flour, respectively, while wheat and oatS represent the comparable water-soluble antigens. Purified antigens such as α-lactalbumin, β-lactoglobulin, casein and ovalbumin (Sigma Chemical Company, St. Louis, MO, USA) were solubilized in 0.02 M ammonium acetate (AmAc), pH 7.0, while crude antigens were extracted with AmAc from wheat, oatS, soy, pork meat or codfish. The antigen preparations were frozen and thawed several times during the extraction procedure to increase the protein output.

Antibody reagents for immunoassays

Isotype-specific rabbit antisera to human IgG and IgA were prepared in our own laboratory (LIIPAT Nos. 38 and 252). Rabbit antibody to human IgM (Code No. A 0425) was obtained from DAKO (Glostrup, Denmark). Alkaline phosphatase-conjugated swine anti-rabbit IgG was obtained from Orion Diagnostica (Espoo, Finland). Total immunoglobulin levels in serum and jejunal fluid were determined by enzyme-linked immunosorbent assay (ELISA) as previously described. [18][19] Albumin was measured by a commercial radioimmunoassay (Pharmacia, Uppsala, Sweden).

Measurements of antibody activities

Relative levels of IgG and IgA antibody activities against different food antigens were determined with an ELISA slightly modified from our previous method. [17] Briefly, antigens in 0.02 M AmAc were coated onto Costar microtitre plates No. 3590 (Cambridge, MA, USA) and washed. Activities were determined in triplicates of perfusion samples, diluted 1/5 (IgG) or 1/10 (IgA) in 0.02 M Tris buffer (pH 7.4) containing 0.05% Tween 20 and 0.5% (w/v) bovine serum albumin (BSA) for gliadin, wheat, oatE, oatS, soy, casein, β-lactoglobulin and codfish, or 0.5% (w/v) gelatine for α-lactalbumin, ovalbumin and pork meat, in addition to NaCl (29 g/l) and KCl (0.2 g/l) for both set-ups. The reactions with secondary (rabbit antibody to human IgG or IgA) and tertiary (alkaline phosphatase-conjugated swine anti-rabbit IgG) immunoreagent took place in the same buffers as those used for the respective primary steps. The final step with alkaline

phosphatase substrate took place in diethanolamine buffer (pH 9.6) before reading of optical densities (OD) at 405 nm.

After coating, the ELISA plates were treated with 0.5% BSA in AmAc for 3 hrs; also the successive reaction steps took place in the presence of BSA, which made it possible to store coated plates beneath a moist filter paper in the refrigerator for a couple of weeks. With gelatine, however, the plates were preferably used immediately after coating. Deionized water was used for washing after the coating and the blocking steps. For washings between sequential antibody reaction steps, Tween 20 (500 μl/l) was added to the water to inhibit unspecific binding of proteins to the plates. It was ensured that the substances added to the perfusion fluid did not interfere with the measurements.

IgM antibody activities to the same antigens were measured both in serum (1/400) and intestinal fluid (1/10) as described above for IgG and IgA, with the following exceptions: The first step (test sample) was reduced from overnight to 2 hrs while the concentration of BSA was increased to 2.5% (w/v) and gelatine to 1% (w/v) because unspecific binding is problematic when IgM antibodies are determined in serum. Blocking with BSA was omitted after coating with soy and both oats preparations. With codfish, the gelatine buffer was used instead of the BSA buffer.

The results were expressed in units per ml (U/ml) related to a serum pool from patients with untreated coeliac disease, [17] arbitrarily taken to contain 1000 U/ml of IgG and IgA antibodies and 250 U/ml of IgM antibodies, thus providing incomparable isotype and specificity values. Standard curves were constructed from serial dilutions of the reference serum, and sample readings were performed by an ELISACalc data programme developed in our laboratory to provide mathematical curve-fitting. [17]

Statistical evaluation

The Mann-Whitney two-tailed nonparametric test was used for statistical comparisons with $p<0.05$ as significance level. Because the positive results obtained generally appeared to be mutually correlated as shown by Spearman correlations analyses (see later), full Bonferroni adjustment for multiple tests would be too conservative (http://home.clara.net/sisa/bonhlp.htm). The p-values should probably be adjusted somewhere between no correction or full; both these extremes are given in the tables, because it is not possible to know exactly to what extent the data must be corrected. Notably, however, full Bonferroni correction did not alter the main conclusions of the study.

Table 1 Total levels (mg/L) of immunglobulins, including secretory IgA (SIgA) in jejunal perfusion fluid from patients with rheumatoid arthritis and healthy control subjects

Subjects	IgA	SIgA	IgM	IgG
Patients				
Median	33	25	5.4	5.2
Range	11-70	13-50	0.3-9.0	1.9-14
Quartile	14; 42	13; 36	3.0; 7.8	4.1; 7.1
Controls				
Median	24	20	1.6	5.1
Range	5.0-56	2.8-44	0.3-7.3	1.1-10.8
Quartile	14.6; 31	11.7; 28	0.8; 2.3	2.5; 7.5
Probability	p<0.33	p<0.20	p<0.0003	p<0.51

RESULTS

Total immunoglobulin levels and food antibodies

The total level of IgM in jejunal fluid of RA patients was highly significantly increased, whereas that of IgA and secretory IgA (SIgA) only showed a trend towards elevation (table 1). The median intestinal level of IgG was the same in RA patients as in healthy control subjects, attesting to the notion that NSAID treatment had not caused any general increase in mucosal permeability for intact proteins (see below).

Jejunal IgA, IgG, and IgM activities to nearly all test antigens were highly or moderately increased in RA patients when compared with controls by ranking of accumulated antibody levels (figs 1 and 2A). Especially the IgM activities were strikingly raised, and this elevation was unrelated to the total IgM levels (fig 2A). The antibody increases were generally significant or highly significant, even after full Bonferroni correction (table 2). Exceptions were IgM and IgG activities against β-1actoglobulin, and IgG activities against α-lactalbumin, ovalbumin and soy. Likewise, intestinal IgA activity against gliadin and casein showed no convincing increase (table 2).

Table 2 Relative IgA, IgG and IgM activity levels (ELISA units/ml, median and range) to various food antigens in jejunal perfusion fluid from patients with rheumatoid arthritis and healthy control subjects

Antigens	Patients	Controls	Probability[a]
		IgA	
Gliadin	5.3 (1.3-75)	2.8 (0.5-20.0)	p=0.068 (p=0.68)[d]
OatE[b]	7.0 (2.5-25.0)	3.8 (0.4-9.7)	p=0.029 (p=0.29)
OatS[c]	2.1 (0.9-9.3)	0.9 (0.3-3.8)	p=0.0026 (p=0.026)
Soy	2.2 (0.7-6.2)	1.0 (0.1-5.8)	p=0.027 (p=0.27)
β-lactoglobulin	2.1 (0.9-6.3)	0.8 (0.1-2.3)	p<<0.0004 (p<0.004)
α-lactalbumin	4.6 (1.5-21.0)	1.8 (0.8-5.5)	p=0.0086 (p=0.086)
Casein	2.0 (0.3-5.7)	1.5 (0.1-7.5)	p=0.301 (p=1.0)
Ovalbumin	2.7 (0.9-11.0)	1.7 (0.1-4.8)	p=0.016 (p=0.16)
Pork meat	8.8 (3.3-25.0)	2.4 (0.3-7.5)	p=<<0.0004 (p=<0.004)
Codfish	5.3 (1.7-10.0)	1.9 (0.3-7.5)	p=0.0004 (p=0.004)
		IgG	
Gliadin	1.1 (0.3-4.5)	0.09 (0.03-1.1)	p<0.0004 (p<0.004)
OatE[b]	1.0 (0.3-3.0)	0.20 (0.03-0.9)	p<0.0004 (p<0.004)
OatS[c]	0.3 (0.03-1.1)	0.04 (0.03-0.8)	p=0.0086 (p=0.086)
Soy	0.4 (0.03-1.4)	0.08 (0.03-0.7)	p=0.0147 (p=0.147)
β-lactoglobulin	0.3 (0.03-1.9)	0.16 (0.03-0.6)	p=0.149 (p=1.0)
α-lactalbumin	0.7 (0.03-2.8)	0.15 (0.04-1.7)	p=0.086 (p=0.86)
Casein	0.5 (0.04-1.9)	0.04 (0.03-0.9)	p=0.0028 (p=0.028)
Ovalbumin	0.7 (0.04-4.3)	0.16 (0.03-1.8)	p=0.023 (p=0.23)
Pork meat	1.5 (0.1-5.8)	0.27 (0.04-0.4)	p=0.0006 (p=0.006)
Codfish	0.9 (0.07-3.5)	0.18 (0.03-0.6)	p=0.0004 (p=0.004)
		IgM	
Gliadin	10.9 (3.6-45.2)	3.0 (0.7-12.8)	p<0.0004 (p<0.0036)
OatE[b]	Not done	Not done	
OatS[c]	7.8 (3.5-33.7)	2.7 (1.2-6.8)	p<<0.0004 (p<0.0036)
Soy	5.3 (1.8-21.8)	1.7 (1.3-3.1)	p<0.0004 (p<0.0036)
β-lactoglobulin	3.8 (0.5-24.8)	1.2 (0.5-4.1)	p=0.031 (p=0.279)
α-lactalbumin	5.3 (2.3-37.1)	1.4 (0.5-2.3)	p<<0.0004 (p<0.0036)
Casein	4.3 (2.3-30.5)	0.6 (0-2.1)	p<<0.0004 (p<0.0036)
Ovalbumin	3.5 (1.6-26.8)	0.5 (0-1.3)	p<<0.0004 (p<0.0036)
Pork meat	9.8 (3.1-34.9)	2.9 (0.4-20.0)	p=0.0014 (p=0.0126)
Codfish	8.8 (3.0-40.0)	2.8 (0.1-22.0)	p=0.0016 (p=0.0144)

[a]The probability that food-specific lgA, IgG or IgM activity is increased, compared with matched controls, according to the Mann-Whitney two-tailed test.
[b]OatE: Oats flour extracted with 70% ethanol.
[c]OatS: Oats flour extracted with ammonium acetate.

[d]P-values in parentheses are subjected to full Bonferroni adjustment for multiple tests.

Interestingly, the two latter food proteins were the only antigens against which the serum IgM activity was significantly raised in RA (fig 2B and table 3), perhaps reflecting abundant antigen uptake due to lack of a substantial mucosal IgA response. Also, serum and intestinal IgM activities to these two antigens were not correlated (r=0.02); only IgM directed against β-1actoglobulin (r=0.75, p=0.0005), oatS (r=0.55, p=0.023) and α-lactalbumin (r=0.54, p=0.025) showed some relationship in the two body fluids (uncorrected p-values). Notably, the serum IgM activities to casein and ovalbumin tended to be correlated with the intestinal albumin level (r=0.67, p<0025 and r=0.59, p<0.05, respectively), possibly supporting the idea that systemic immune activation depends on an inadequate mucosal barrier function. Conversely, the intestinal IgM activities to all antigens were statistically unrelated to any excessive leakage of albumin into the lumen (data not shown); and, as mentioned above, there was no indication of a generally increased intestinal permeability for proteins in the RA patients because the average jejunal IgG level was normal (table 1).

Table 3 Relative IgM activity levels (ELISA units/ml, median and range) against various food antigens in serum samples from patients with rheumatoid arthritis and healthy control subjects

Antigens	Patients	Controls	Probability[a]
Gliadin	597 (104-1087)	239 (651-1396)	p<0.0004 (p<0.0036)[c]
OatS[b]	270 (36-627)	136 (25-575)	p=0.057 (p=0.513)
Soy	159 (34-403)	131 (39-774)	p=0.575 (p=1.0)
β-lactoglobulin	61 (10-344)	32 (5-101)	p=0.074 (p=0.666)
α-lactalbumin	46 (2-103)	52 (18-158)	p=0.189 (p=1.0)
Casein	191 (43-523)	46 (1-204)	p<<0.0004 (p<0.0036)
Ovalbumin	71 (12-121)	49 (1-130)	p=0.18 (p=1.0)
Pork meat	54 (31-98)	109 (34-159)	p=1.0
Codfish	84 (35-200)	123 (39-381)	p=1.0

[a]The probability that food-specific IgM activity is increased, compared with matched controls, according to the Mann-Whitney two-tailed test.
[b]OatS: Oats flour extracted with ammonium acetate.
[c]P-values in parentheses are subjected to full Bonferroni adjustment for multiple tests.

Antibody activities to food antigens are both related and unrelated

Because food intake and disease severity show an apparent connection in only some RA patients, we identified those with elevated intestinal antibody activities. In the control group, IgA activities to most food antigens correlated with the total intestinal IgA levels. Therefore, we drew a linear regression line for total IgA and IgA activity in controls and considered RA activities to be increased when they were above the limiting control lines as suggested by Karol et al. [20] for serum IgE antibodies. However, IgG and IgM activities as well as IgA antibodies to flour antigens (including soy) did not show regression with the total isotype levels; for these activities the mean values for controls plus two standard deviations were considered the upper limit when recording an antibody increase in RA patients.

The proportion of patients showing antibody increase depended both on the Ig class and on the type of food antigen. Thus, patients deemed to have elevated intestinal IgA activity varied for flours from 7% (soy) to 21% (gliadin, OatS), for cow's milk proteins from none (casein) to 50% (α-lactalbumin) or 57% (β-lactoglobulin), and for other types of protein from 28% (ovalbumin) to 71% (cod fish) or 100% (pork meat). Increased IgG activity was less common and varied from 14-21% (α-lactalbumin, β-lactoglobulin, casein, ovalbumin, soy, oats) to 36-57% (pork meat, cod fish, gliadin). IgM activities were often

elevated, varying from 36-50% (pork meat, cod fish, gliadin, β-1actoglobulin) to 57-86% (soy, OatS, α-lactalbumin, casein, ovalbumin).

Individual intestinal IgM activities to different antigens were positively correlated in the RA patients (r=0.68-0.99) and also in the controls, although of lower magnitude (r=0.33-0.93). IgG activities showed positive correlations for most antigens both in RA patients (r=0.68-0.97) and controls (r=0.51-0.99), but usually of lower magnitude than for IgM activities in the patients. IgA activities did not correlate in the RA patients except against some antigens: pork meat *vs.* α-lactalbumin (r=0.53), β-lactoglobulin (r=0.61), soy (r=0.67), cod fish (r =0.74) and ovalbumin (r=0.90); α-lactalbumin *vs.* ovalbumin (r=0.55), casein (r=0.57) and oatE (r=0.73); and oatE *vs.* gliadin (r=0.74). Conversely, IgA activities to most antigens were significantly correlated with each other in the controls. Intestinal antibodies to one and the same antigen did generally not correlate so well among the three Ig classes as did the activities of a specific isotype against different antigens, with the exception of antibodies to gliadin (r=0.85). Otherwise the relationship among different isotypes varied considerably (r=0.06-0.61).

Effect of sulfasalazine

Treatment of five RA patients with SSZ for 16 weeks reduced the elevated food antibody levels initially seen in three of them; this effect was striking in the two with the highest levels (fig 3). Immunosuppression exerted by SSZ on the intestinal immune responses was thus suggested. It has also been proposed that this drug can diminish mucosal permeability, but luminal albumin levels were not consistently decreased after the treatment – regardless of whether the patients had received NSAIDs recently or not (fig 4). Neither was the antibody decrease accompanied by any apparent reduction of total Ig levels in jejunal fluid (data not shown), which was in contrast to our observations after SSZ treatment in a contemporary study of patients with ankylosing spondylitis. [19] In that disease, the suppressive effect on the intestinal IgM ($p<0.01$) and SIgA ($p<0.002$) levels was accompanied by clinical improvement and reduced ESR ($p<0.004$). [19] In the few RA patients subjected to SSZ treatment, however, the clinical effect was associated with reduced ESR but not necessarily with decreased jejunal antibody production (fig 3). Because a high protein intake gives an OR of only 2.9 for polyarthritis, [3] a much larger study group would clearly be required to demonstrate convincingly and association between intestinal food antibodies and severity of RA.

Cross-reactivity of food antibodies revealed by absorption test

Varying amounts of the coating antigen (1.56-10 μg/ml) added to intestinal fluid the day before ELISA performance, had generally no effect on the readings, or could even increase them – probably because of the formation of soluble immune complexes which remained able to react with the coat. We therefore performed antibody absorption with a large excess of gliadin that is insoluble in aqueous solution. Thus, intestinal fluid (fig 5A) and serum (fig 5B) samples were tested in ELISA for remaining IgA and IgM antibody activities after being mixed with gliadin (0.1 g/ml) overnight on a shaker at room temperature. Residual IgM activity against gliadin was only 0%-18% and against unrelated antigens 50-85%. Remaining IgA activity was measured only in one intestinal fluid (fig 5A); with most antigens the proportion of residual antibody activity was lower than that for IgM.

DISCUSSION

This is the first extensive study of intestinal food antibodies in RA patients. Despite considerable variability, which can be expected for antibody levels even in healthy adults regardless of age and sex, [21] our results were remarkably positive in RA – particularly for

the IgM class – and included in a surprising manner most test antigens. Absorption with insoluble gliadin revealed a substantial level of cross-reactivity for both IgM and IgA antibodies. Notably, even in the normal state, SIgA in various human secretions has been reported to exhibit a relatively high level of cross-reactivity, recognizing both self and microbial antigens, [22] but apparently without involving peritoneally derived B1 cells, in contrast to the situation in mice. [23] It rather reflects a substantial innate drive of the intestinal immune system. [23]

The IgM reactivity in jejunal fluid was only marginally related to that in serum of the same patient, and it was neither related to total IgM levels nor to mucosal protein permeability as deemed by mucosal leakage of albumin or IgG. Therefore, a truly RA-related mucosal production of antibodies was strongly suggested, rather than excessive immunostimulation after potentially NSAID-induced absorption of dietary antigens. [12] Also notably, two patients that had not received NSAIDs for at least 1 month showed elevated intestinal IgM and IgA food reactivity as well. Increased levels of circulating SIgA associated with IgA RF complexes have been observed in RA patients, [11] likewise suggesting that intestinal immunity is overactivated in this disease.

RA sera are known to contain increased amounts of so-called 'natural antibodies', which are encoded by germ-line Ig variable genes with only few or no somatic mutations. Such antibodies display a broad array of mostly autoimmune activity, perhaps enabling them to clear waste products. [24] In serum, this activity has been thought to represent low-avidity IgM antibodies but is, instead, mainly of the IgG class. [24] RF is mostly of the IgM, IgG or IgA class [25] and appears to be subjected to antigen-driven mutation in RA, but still exhibiting substantial cross-reactivity like other autoantibodies. [26] Therefore, it cannot be excluded that food antigens are involved in the RF induction process, according to the 'multiple hit model' for RA. [27] The underlying immunoregulatory defect may involve both poor activity of regulatory T cells [28] and impaired early B-cell tolerance. [29]

Polyreactive antibodies most likely have a flexible antigen-binding 'pocket' that can accommodate different antigens. [30] Polyreactivity of RF could hence explain the overall tendency to increased IgM activities in RA serum irrespective of test antigen, for instance *Proteus mirabilis*, staphylococcal enterotoxin B, β_2-microglobulin and cytokines. [31][32][33][34] It should also be noted that investigation of food antibodies in RA sera is difficult because RF of different Ig classes may cause assay interference. [35] Another complication is that lgM RF binds IgG from other species, including the antibodies used in the assay. [36] A panel of food antigens to document gut antibody cross-reactivity in RA has apparently not been used before, although it should be pointed out that we have not demonstrated polyreactivity in the formal sense.

Intestinal IgM and IgA with RF activity have been observed in patients with untreated coeliac disease, and the IgM RF level was quite high in coeliac patients with IgA deficiency. [37] Mucosal RF synthesis is apparently linked to the gluten response because RF in serum from patients with coeliac disease or dermatitis herpetiformis was carried only by IgA. [38] Furthermore, SIgA RF has been detected in serum from RA patients, so some intestinal RF synthesis may take place also in RA. [11] However, we found that intestinal fluid from RA patients contained only low levels of IgA and IgM RF, some 1000 times less than in serum. [15] The IgA, but notably not the IgM, RF activities were generally well correlated with the food antibody levels of all the three Ig classes (r=0.65-0.94; p=0.01-0.0001).

Multispecific antibodies may exist in antigen complexes. [39] In the gut, such complex formation depends on antigen stability and on pH-dependent pepsin hydrolysis. Thus, infants are prone to develop cow's milk allergy while their gastric acidity is pH 3-4 (compared to pH 2 in adults); at pH 4 the degradation of α-lactalbumin, BSA and bovine IgG is markedly reduced in contrast to β-lactoglobulin. [40] Some 80% of untreated RA patients

have been shown to have reduced maximal gastric acid output, [41] which could contribute to enhanced food immunoreactivity.

A germ-free state prevents the development of gut and joint inflammation in HLA-B27 transgenic rats, thereby giving strong support to a connection between mucosal immunity and arthritis. [42] Also, reactive arthritis in humans appears to be caused by a combination of a mucosa-associated microbial impact and genetic predisposition. [43] Interestingly, some 90% of patients with reactive arthritis or ankylosing spondylitis express HLA-B27, and these disorders can be associated with Crohn's disease, ulcerative colitis and jejuno-ileal bypass surgery [43] – again emphasizing the putative gut-joint axis which is also supported by shared homing properties of activated intestinal immune cells. [44]

Moreover, animal experiments have demonstrated a widespread tissue distribution of food antigens shortly after feeding, [45] which could predispose to synovial immune complex formation and thereby autoimmune joint reactions. [27] We have previously reported that intestinal levels of IgM and IgA are increased in patients with ankylosing spondylitis related to disease activity. [19] Antigens from the gut microbiota rather than food are apparently involved in that disease, [43][44] because the IgM reactivity to dietary antigens was not different from normal control levels (our unpublished observations), in striking contrast to the data presented here for RA. Disparate antigenic or mitogenic stimulation in the gut might explain the different response to SSZ treatment noted in the two disorders with regard to reduction of total intestinal immunoglobulin levels.

To conclude, both systemic and intestinal humoral immunity was found to be aberrant in many RA patients, with particularly striking elevation of cross-reactive food antibodies in proximal gut secretions. IgM reactivity against some food items was increased also in serum, but relatively much less so. Measurements of serum antibodies (except for RF) appears to be of little informative value in RA. Conversely, measurements of intestinal antibodies provide more remarkable results, suggesting a connection between mucosal immune activation and the pathogenesis of RA, at least in some patients. Their food-related problems may reflect the additive effect of multiple modest hypersensitivity reactions mediated, for instance, by immune complexes which could predispose the joints for autoimmune tissue-destructive reactions. [27]

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Legends to figures

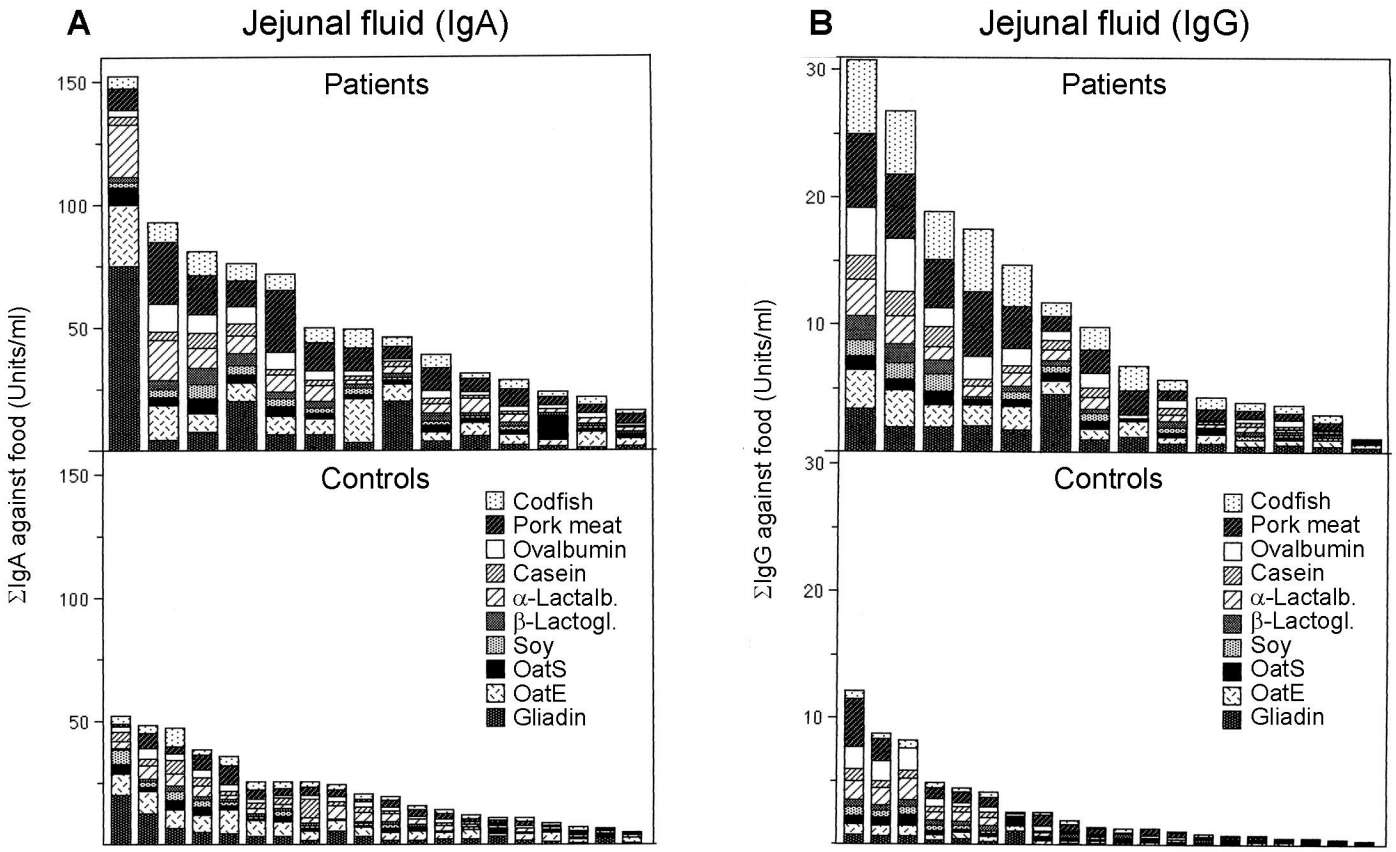
Figure 1 ELISA determinations of IgA (A) and IgG (B) antibody activities against various food antigens as indicated in jejunal perfusion fluid obtained from RA patients (n=14) and healthy controls (n=20). Columns represent accumulative antibody levels (see key), arranged in decreasing order for individual subjects. Note different scales on vertical axes.

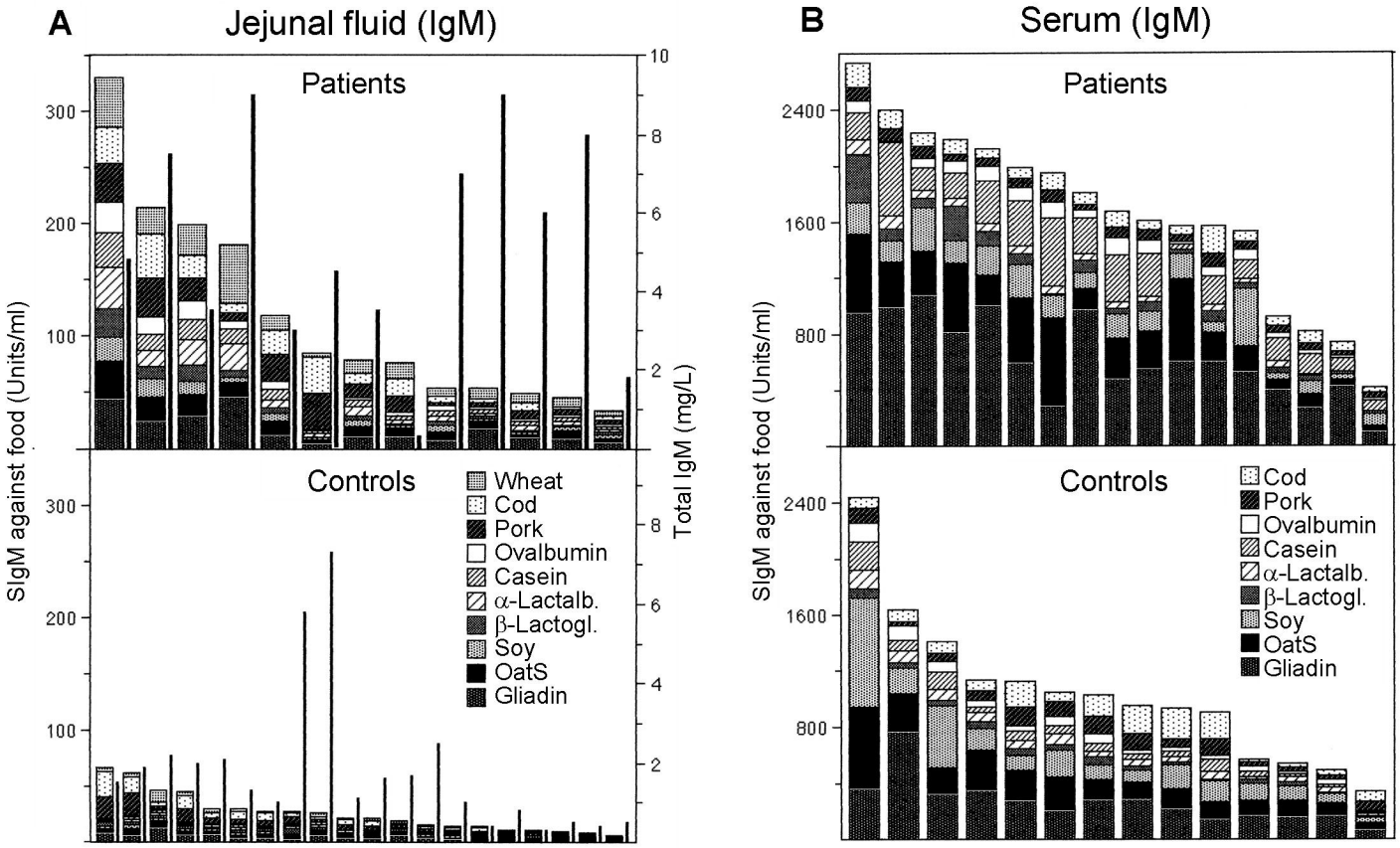
Figure 2 ELISA determinations of IgM antibody activities against various food antigens as indicated in jejunal perfusion fluid (A) obtained from RA patients (n=13) and healthy controls (n=20), and in serum (B) of RA patients (n=17) and healthy controls (n=14). Columns represent accumulative antibody levels (see key), arranged in decreasing order for individual subjects. Thin vertical bars in (A) represent total intestinal IgM concentrations for comparison with the individual IgM antibody levels. Note different scales on the three vertical axes.

Figure 3 ELISA determinations of IgM, IgA and IgG antibody activities against various food antigens as indicated (see key) in jejunal perfusion fluid obtained from five RA patients (P1-P5) before (Be) and after (Af) treatment with sulfasalazine for 16 weeks. P1, P3 and P4 had not received NSAIDs for at least 1 month before sampling (see fig 4). The effect of sulfasalazine on the erythrocyte sedimentation rate (ESR) and clinical improvement (+ to ++) or worsening (-) following the treatment, are indicated at the bottom. Note different scales on vertical axes.

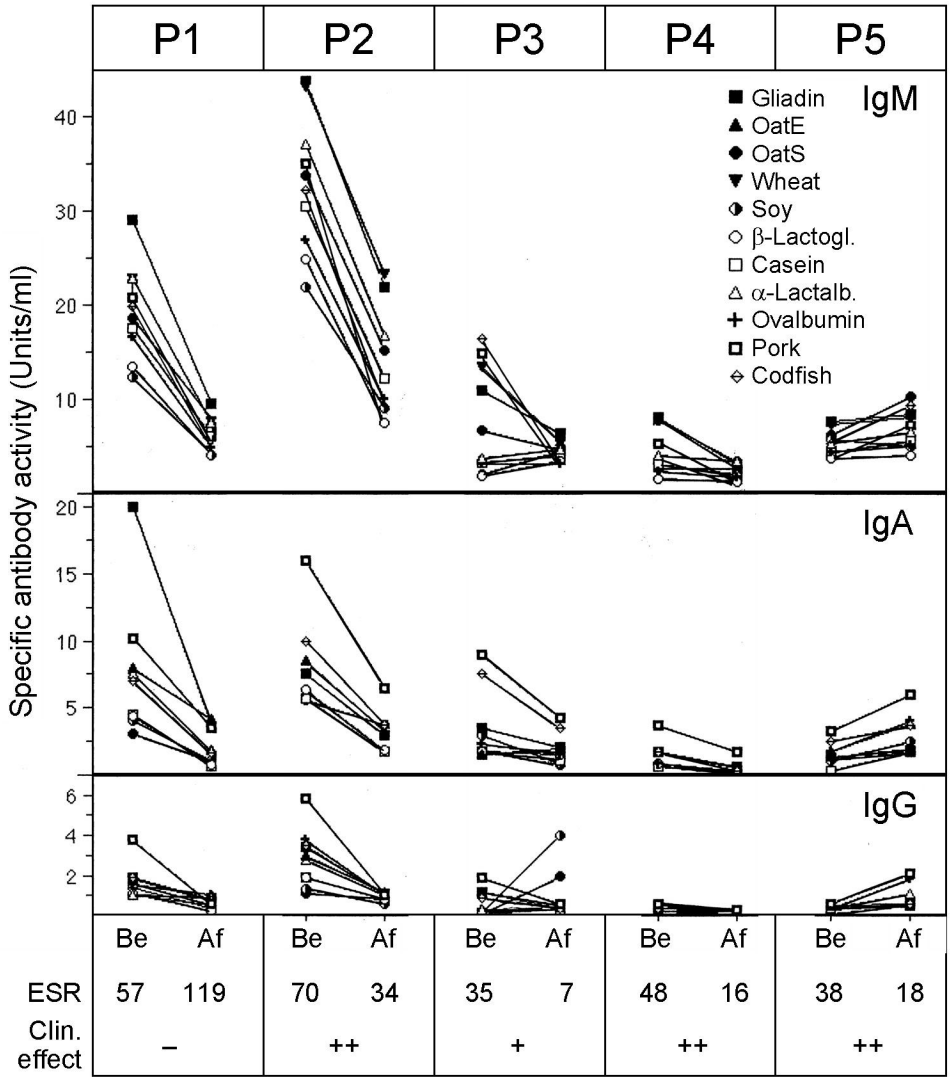
Figure 4 Radioimmunoassay determinations of albumin in jejunal perfusion fluid obtained from the same RA patients (P1-P5) as reported in fig 3; those encircled had not received NSAIDs for at least 1 month. There was no consistent difference in the albumin levels before (Be) or after (Af) sulfasalazine treatment for 16 weeks (medians indicated by horizontal bars).

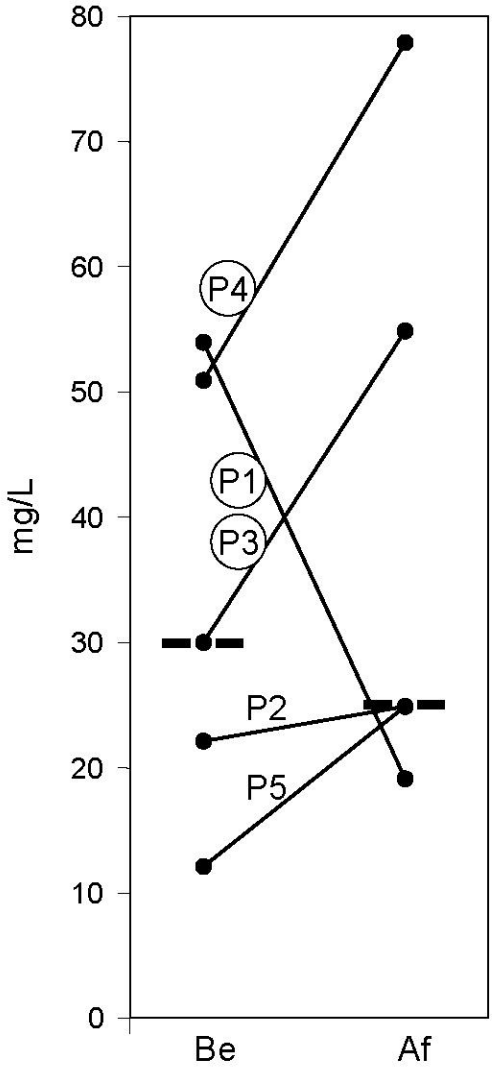
Figure 5 ELISA determinations of IgA and IgM antibody activities (% of original levels) against various food antigens as indicated (see key), remaining in two different perfusion fluids (Perf 451 and Perf 455) obtained from the jejunum (A), and in serum (B), of RA patients after absorption of the samples with an excess of insoluble gliadin.





Hvatum et al. Fig 3.





Hvatum et al. Fig 5.