GUT IMMUNOLOGY

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

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Background and aims: Patients with rheumatoid arthritis (RA) often feel there is an association between food intake and rheumatoid disease severity. To investigate a putative immunological link between gut immunity and RA, food antibodies were measured in serum and perfusion fluid from the jejunum of RA patients and healthy controls to determine the systemic and mucosal immune response.
Methods: IgG, IgA, and IgM antibodies to dietary antigens were measured 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 immunoglobulin classes showed increased food specific activities. Except for IgM activity against β-lactoglobulin, all other IgM activities were significantly increased irrespective of the total IgM level. The RA associated serum IgM antibody responses were relatively much less pronounced. Compared with IgM, the intestinal IgA activities were less consistently raised, 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 normalised after treatment, but clinical improvement was better reflected in a reduced erythrocyte sedimentation rate.
Conclusions: The production of cross reactive antibodies is strikingly increased 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.

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P atients 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 protocols, followed by some sort of food challenge. Food hypersensitivity in RA does not reflect IgE mediated allergy, and most studies have concluded that food is unlikely to have a pathogenic effect in RA. Thus, Panush[2] carried out 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, determining odds ratios (ORs) after adjusting for possible confounding variables, suggested 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]

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 suggest that food does have pathogenic importance, 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 increased IgM activities were found, there was no convincing association with clinical variables or dietetic benefits.[7] Raised 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% concurrently had IgA rheumatoid factor (RF) and there was some association with duodenal villous atrophy.[9] However, a subsequent study was contradictory.[10]

Overall, 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 the serum, and the fact that circulating IgA RF is predominantly polymeric supports a mucosal origin.[11] 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] We therefore 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.

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 to 70) and the mean duration of disease 10 years (range 9 months to 30 years). Twenty healthy subjects served as
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Abbreviations: RA, rheumatoid arthritis; RF, rheumatoid factor; NSAID, non-steroidal anti-inflammatory drug; ELISA, enzyme linked immunosorbent assay; BSA, bovine serum albumin; OD, optical density; SIgA, secretory IgA.

controls; their sex distribution was similar to the RA patients but because of ethical constraints we were unable to match the controls for age, although the ranges were highly overlapping (mean age 29 years; range 23 to 39). RA patients and controls were all white and were taking a normal diet 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 $\geqslant 60$ minutes, tenderness or swelling or both of six or more joints, and an erythrocyte sedimentation rate (ESR) of >30 mm in the first hour. None of the patients had received treatment with gold, penicillamine, chloroquine, sulfasalazine, corticosteroids, or immunosuppressants for the three months before 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 undertaken.

Five of the patients were reinvestigated after treatment with sulfasalazine for 16 weeks; three of these had not received NSAIDs for at least one month. The dose of sulfasalazine was increased by 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 were approved by the local ethics committee. Most of the subjects were included in 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 175 cm long and containing 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 phenolsulphonphthalein (phenol red) as a marker of the patency of the proximal balloon. The recovery of the volume marker was (mean (SD)) 86 (10)% in RA patients and 89 (6)% in controls. The fluid was collected on ice, centrifuged in cold conditions at 2500 $\times g$, 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 oat 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, Missouri, USA) were

solubilised in 0.02 M ammonium acetate, pH 7.0, while crude antigens were extracted with ammonium acetate 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 A0425) 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 ammonium acetate were coated onto Costar microtitre plates, No 3590 (Cambridge, Massachusetts, 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% (wt/vol) bovine serum albumin (BSA) for gliadin, wheat, oatE, oatS, soy, casein, β-lactoglobulin, and codfish, or 0.5% (wt/vol) gelatin for α-lactalbumin, ovalbumin, and pork meat, in addition to NaCl (29 g/l) and KCl (0.2 g/l) for both procedures. The reactions with secondary (rabbit antibody to human IgG or IgA) and tertiary (alkaline phosphatase conjugated swine anti-rabbit IgG) immunoreagents 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 ammonium acetate for three hours; 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. Deionised 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 non-specific 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 two hours, while the concentration of BSA was increased to 2.5% (wt/vol) and gelatine to 1% (wt/vol) because non-specific binding is a problem when IgM antibodies are determined in serum. Blocking with BSA was omitted after coating with soy and both of the oats preparations. With codfish, the gelatine buffer was used instead of the BSA buffer.

The results are 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 carried out using an ELISACalc

Table 1 Total levels (mg/l) of immunglobulins, including secretory IgA (SIgA) in jejunal perfusion fluid

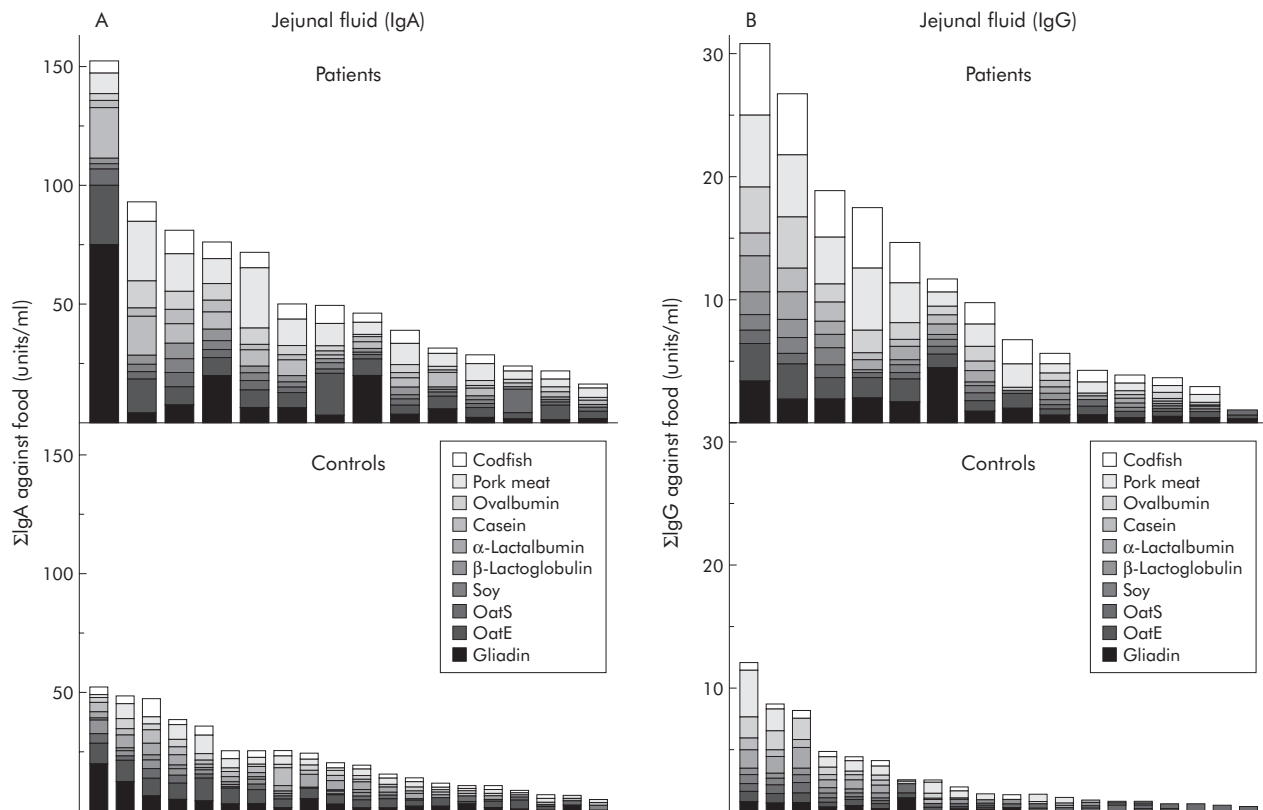| Subjects	IgA	SIgA	IgM	IgG
RA patients				
Median	33	25	5.4	5.2
Range	11 to 70	13 to 50	0.3 to 9.0	1.9 to 14
Quartile	14; 42	13; 36	3.0; 7.8	4.1; 7.1
Healthy controls				
Median	24	20	1.6	5.1
Range	5.0 to 56	2.8 to 44	0.3 to 7.3	1.1 to 10.8
Quartile	14.6; 31	11.7; 28	0.8; 2.3	2.5; 7.5
Probability	p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

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

data program developed in our laboratory to provide mathematical curve fitting.[17]

Statistical evaluation

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

RESULTS

Total immunoglobulin levels and food antibodies

The total level of IgM in jejunal fluid of RA patients was significantly increased, whereas that of IgA and secretory IgA (SIgA) only showed a trend towards an increase (table 1). The median intestinal level of IgG was the same in RA patients as in healthy control subjects, supporting the view 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). In particular, 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).

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 owing 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 relation 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).

Antibody activities to food antigens are both related and unrelated

Because food intake and disease severity show an apparent connection in some RA patients only, we identified those with increased intestinal antibody activities. In the control group, IgA activities to most food antigens correlated with
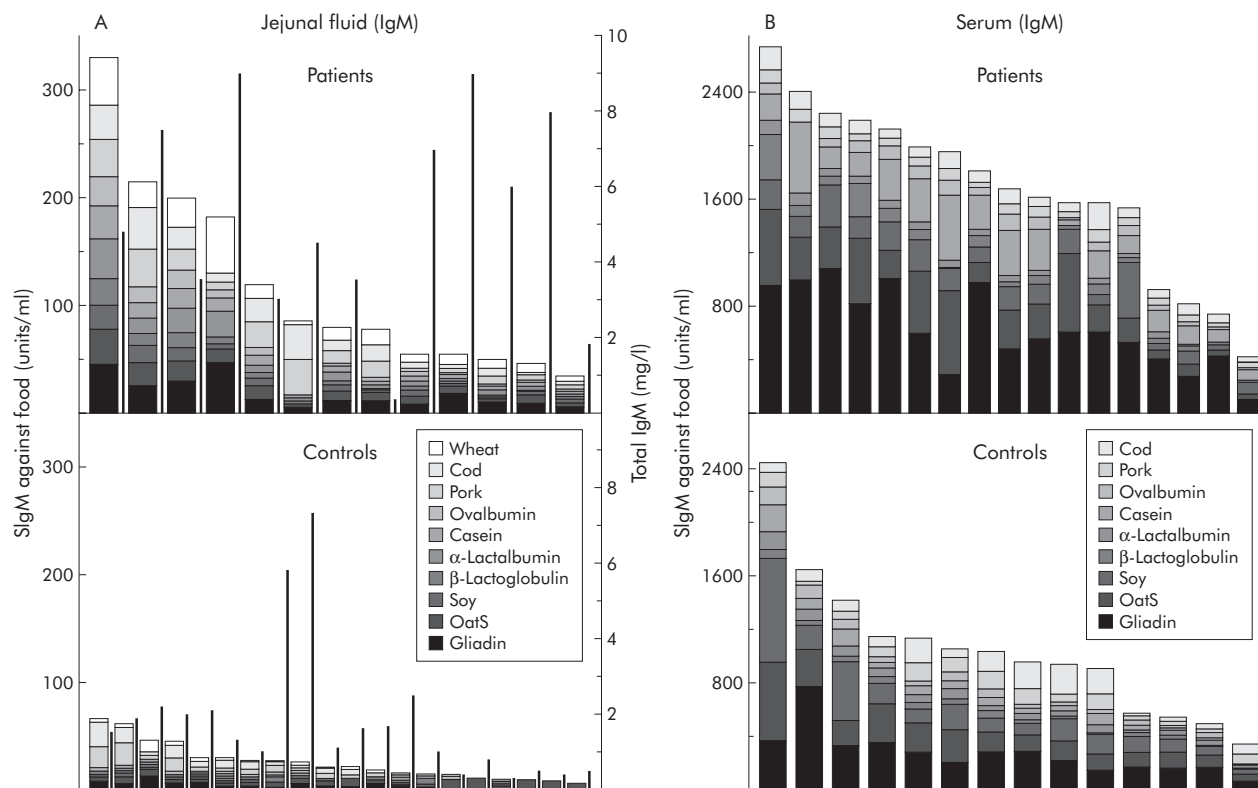
Figure 2 Enzyme linked immunosorbent assay (ELISA) determinations of IgM antibody activities against various food antigens (see key) in jejunal perfusion fluid (A) obtained from patients with rheumatoid arthritis (patients) (n = 13) and healthy controls (n = 20), and in serum (B) from patients (n = 17) and controls (n = 14). Columns represent accumulative antibody levels, 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.

the total intestinal IgA levels. We therefore 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 increased 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 increased, 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 ($r = 0.68$ to 0.99) and also in the controls, although at lower magnitude ($r = 0.33$ to 0.93). IgG activities showed positive correlations for most antigens both in RA patients ($r = 0.68$ to 0.97) and controls ($r = 0.51$ to 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 *v* 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 *v* ovalbumin ($r = 0.55$), casein ($r = 0.57$), and oatE ($r = 0.73$); and oatE *v* 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 generally did 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 relations among different isotypes varied considerably ($r = 0.06$ to $r = 0.61$).

Effect of sulfasalazine
Treatment of five RA patients with sulfasalazine for 16 weeks reduced the increased food antibody levels seen initially in three of them; this effect was striking in the two with the highest levels (fig 3), suggesting that sulfasalazine had an immunosuppressive effect on intestinal immune responses. 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)—in contrast to our observations after sulfasalazine 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 sulfasalazine treatment, however, the clinical effect was associated with reduced ESR but not necessarily with decreased jejunal

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

*The probability that food specific IgA, IgG, or IgM activity is increased compared with matched controls (Mann–Whitney two tailed test).
†p Values in parentheses were subjected to full Bonferroni adjustment for multiple tests.
ELISA, enzyme linked immunosorbent assay; OatE. oat flour extracted with 70% ethanol; OatS, oat flour extracted with ammonium acetate.

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 an association between intestinal food antibodies and the severity of RA.

Cross reactivity of food antibodies revealed by absorption test

Varying amounts of the coating antigen (1.56 to 10 μg/ml) added to intestinal fluid the day before the ELISA generally had 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 carried out antibody absorption with a large excess of gliadin, which is insoluble in aqueous solution. Thus intestinal fluid (fig 5A) and serum samples (fig 5B) 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

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

*The probability that food specific IgM activity is increased compared with matched controls (Mann–Whitney two tailed test).
†p Values in parentheses were subjected to full Bonferroni adjustment for multiple tests.
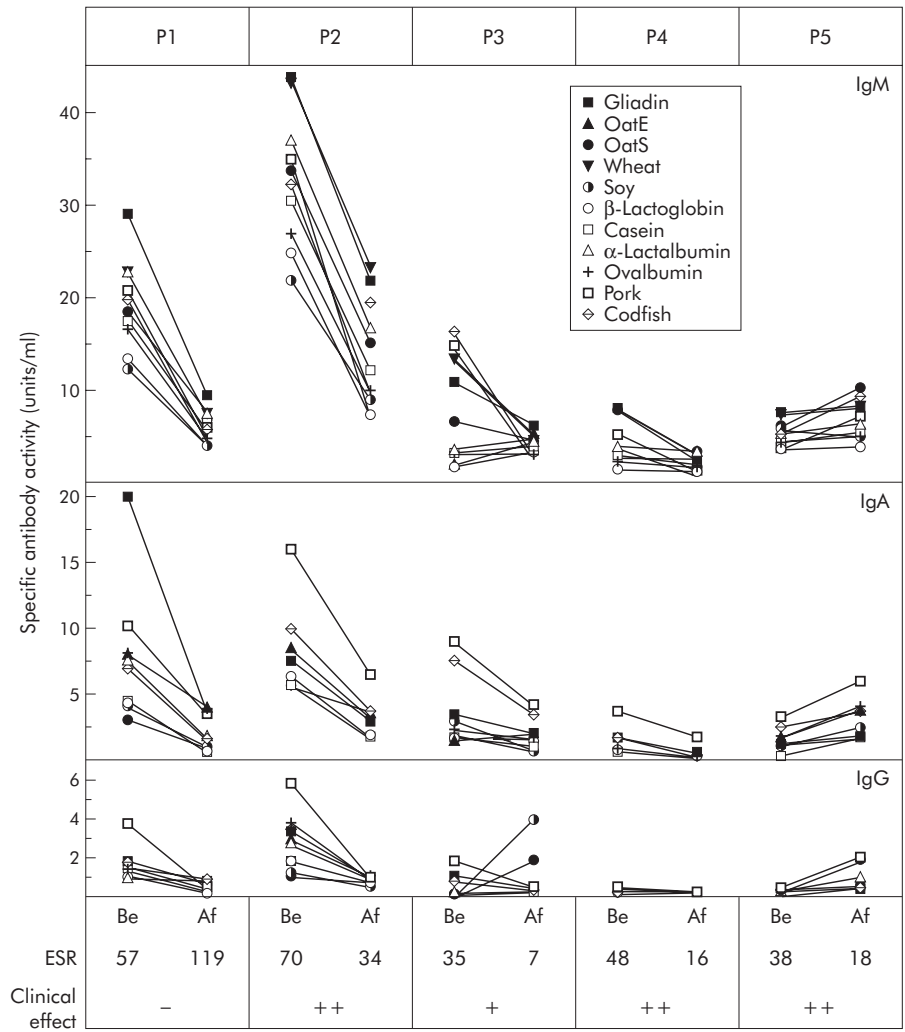ELISA, enzyme linked immunosorbent assay; OatS, oat flour extracted with ammonium acetate.

Figure 3 Enzyme linked immunosorbent assay determinations of IgM, IgA, and IgG antibody activities against various food antigens as indicated (see key) in jejunal perfusion fluid obtained from five patients with rheumatoid arthritis (P1–P5) before (Be) and after (Af) treatment with sulfasalazine for 16 weeks. P1, P3, and P4 had not received NSAIDs for at least one 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 is indicated at the bottom. Note different scales on vertical axes. NSAID, non-steroidal anti-inflammatory drug.
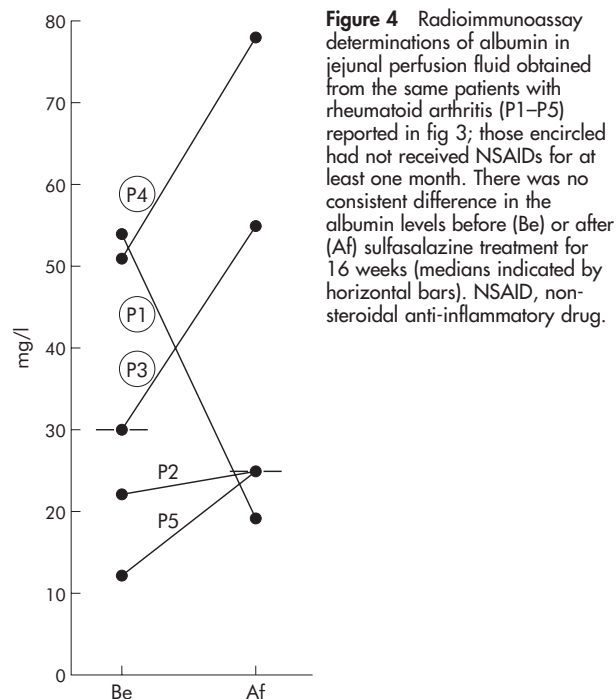

Figure 4 Radioimmunoassay determinations of albumin in jejunal perfusion fluid obtained from the same patients with rheumatoid arthritis (P1–P5) reported in fig 3; those encircled had not received NSAIDs for at least one month. There was no consistent difference in the albumin levels before (Be) or after (Af) sulfasalazine treatment for 16 weeks (medians indicated by horizontal bars). NSAID, non-steroidal anti-inflammatory drug.

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 most test antigens in a surprising manner. 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 show a relatively high level of cross reactivity, recognising both self and microbial antigens,[22] but apparently without involving peritoneally derived B1 cells, in contrast to the situation in mice.[23] Rather, it 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
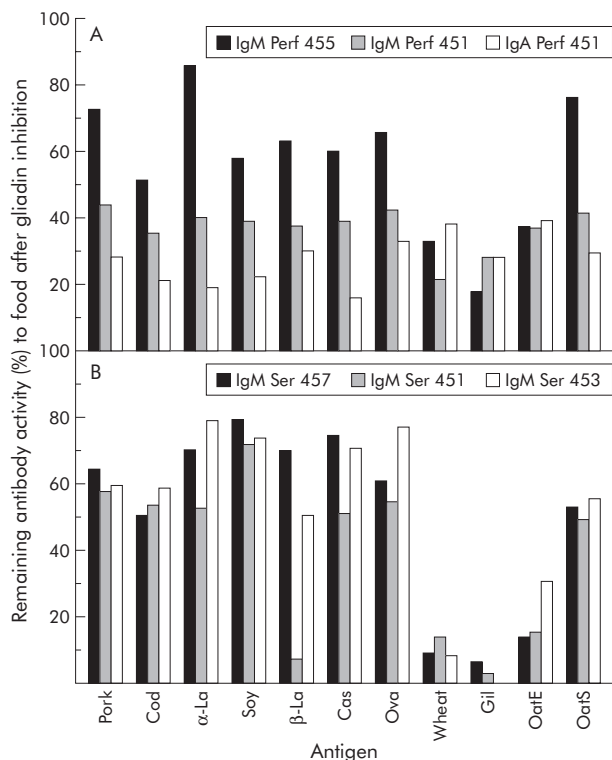
Figure 5 Enzyme linked immunosorbent assay determinations of IgA and IgM antibody activities (per cent of original levels) against various food antigens as indicated (see key), remaining in two different perfusion fluids (Perf 451 and Perf 455) obtained from the jejunum (A) and in three serum samples (B) of patients with rheumatoid arthritis after absorption of the samples with an excess of insoluble gliadin.

immunostimulation after potentially NSAID induced absorption of dietary antigens.[12] Also, notably, two patients who had not received NSAIDs for at least one month showed increased 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 showing substantial cross reactivity, like other autoantibodies.[26] Therefore, it cannot be excluded that food antigens are involved in the rheumatoid factor 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 probably 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.[31–34] 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 IgM 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 ($r = 0.65$ to 0.94; $p = 0.01$ to 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 with 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 maximum 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 emphasising 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 sulfasalazine treatment noted in the two disorders with regard to reduction of total intestinal immunoglobulin levels as mentioned in Results.

Conclusions
Both systemic and intestinal humoral immunity was found to be aberrant in many RA patients, with a 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 striking 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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