Acute and chronic immunological response to dietary antigen

P J GALLAGHER, N J GOULDING, M J GIBNEY, D B JONES, AND JANE MORGAN

SUMMARY On separate mornings at 14 day intervals groups of six to eight healthy fasting male volunteers drank a 5.5 MJ test meal containing 20% milk, egg or soya protein, or a control protein free preparation. Using a Clq binding test, marked but transient rises in circulating immune complexes were detected 30–120 minutes after the milk and egg meals but not with soya or the control. No such changes were seen when complexes were measured by Raji cell immunoassay. Food antigen specific antibodies were present in the plasma of all subjects but showed no consistent pattern of variation in the postprandial period. In most volunteers a chronic increase in milk (three weeks) or soya (six weeks) consumption produced no changes in circulating immune complexes or antibodies to dietary protein. Although two of 16 milk and six of 52 soya volunteers had substantial rises of one or more classes of plasma food antigen specific antibodies they were matched by a similar number in which a decrease was recorded. These results indicate that the formation of circulating immune complexes may be a physiological response to a large load of dietary antigen but that in most adults a chronic increase of milk or soya consumption does not affect food antibody or immune complex concentrations.

At the turn of the century a series of ingenious human and animal1 2 experiments showed that unaltered food protein can reach the systemic circulation in small amounts. More recent studies have shown immunoreactive dietary antigen in both the mesenteric lymphatics3 and the portal venous system and suggest that up to 2% of food protein is absorbed in this way.4 5 Despite this continuous antigenic challenge most adults have only very low levels of circulating antibodies to common dietary proteins such as casein, ovalbumen, or gluten.6 7 Antibody levels may be increased, however, in coeliac disease or severe ulcerative colitis7 and some studies have suggested that antibodies to certain constituents of cow's milk are raised in patients with ischaemic heart disease.8 9 Because of the potential importance of systemic immune reactions to dietary antigens we studied the response to both animal and vegetable proteins on both an acute and chronic basis. Particular attention was paid to variations in the concentrations of plasma food antigen specific antibodies and circulating immune complexes.

Methods

SUBJECTS Experiment 1 Acute feeding At 14 day intervals, after an overnight fast, groups of eight healthy male volunteers, aged 21–42 years, drank a 500 ml test meal containing soya, milk, or egg protein or an isoenergetic control protein free preparation (Table 1). Food antibodies and circulating immune complexes were then estimated in venous blood samples taken at 0, 30, 60, 90, 120, and 180 minutes. Antigen specific antibodies were measured by an enzyme linked immunosorbent assay (ELISA). The assays were performed on polystyrene microtitre plates which had been coated with antigen (dried skimmed milk, hen ovalbumen, or a freeze dried extract of promine D) by overnight incubation at 4°C with 210 µl per well of a 10 µg/ml solution of test protein in pH 9.6 carbonate buffer. Between all stages of the assay plates were thoroughly washed with 0.9% saline containing, like all subsequent
buffering solutions, 0·05% Tween 20. Twenty microlitres of diluted test serum was added for three hours at room temperature. A 1:500 dilution of commercial IgA, IgG, or IgM rabbit antihuman antibody conjugated with horseradish peroxidase was then incubated for two hours. The subsequent assay mixture contained 1 ml of 1% aqueous hydrogen peroxide (substrate) and 0·85 ml of 1% dianisidine hydrochloride (chromogen) per 100 ml of pH 6·0 phosphate buffer. The reaction was read at one hour in a Dynatech plate reading spectrophotometer at 450 nm. The results were expressed as a product of the optical density reading and the serum dilution, corrected to a standard enzyme activity. The coefficient of variation of replicate analyses was always less than 10%.

Circulating immune complexes were measured by both Clq binding and Raji immunoassay, with minor modifications. The Clq binding test measures the ability of immune complexes to bind to radiolabelled purified Clq. Bound complexes are precipitated with 3% polyethylene glycol and the radioactivity of the centrifuged precipitate is expressed as the per cent of the activity of the same amount of labelled Clq precipitated with 20% trichloroacetic acid. The upper limit of normal was taken as 5% and the coefficients of variation of replicate analyses ranged between 6·5 and 23·3%.

In the Raji immunoassay cultured lymphoblastoid cells, rich in complement receptors but free of native surface Ig, are incubated with the test serum and a source of complement. Immune complexes which have activated complement bind to the cell surface and can be assayed with labelled antihuman immunoglobulin antibodies. Alkaline aggregated immunoglobulin was used as a standard and the results expressed as the amount of AHG which would produce the same result as the test serum. The upper limit of normal was taken as 41 μg AHG, the mean plus two standard deviations (15±13) of a group of 65 normal subjects. The coefficients of variation of replicate analyses was less than 20%.

**Experiment 2: Chronic** Thirty-nine male and 27 female volunteers, range 20–68 years, ate at least four meals per week based on textured vegetable protein, for 44 days. The products used included mince meat extenders, prepared dinners (goulash, curry, stroganov, etc), and a soya protein based milk. Sixteen different subjects (10 men and six women, range 21–42 years) increased their daily milk consumption for 21 days. They drank at least 700 ml milk (46 g protein) and 300 g commercial fruit flavoured yoghurt per day, although some drank 500 ml of a concentrate containing 192 g of dried skimmed milk instead.

In the soya experiment all volunteers completed a 24 hour dietary recall questionnaire before, and 35 of the 54 a detailed three day weighed food survey towards the end of the six weeks. In the milk experiment the 16 subjects and seven additional controls wrote up a three day dietary record before beginning the project. Mean daily nutrient intakes were then determined using computerised food tables. The programme used provided details of total energy intake, proportions of fat, carbohydrate and protein, and allowed individual food stuffs such as milk and soya protein to be calculated separately.

Fasting blood samples were taken at the start and finish of each experiment. Immune complexes were measured by the methods used in Experiment 1. Antibodies to milk protein were measured by ELISA in polystyrene microtitre plates but 64 × 10 mm diameter test tubes were used for the soya antibody assays. Total cholesterol and triglycerides were estimated in a Technicon autoanlyser and HDL cholesterol values by heparin-manganese chloride precipitation.

**Results**

**Experiment 1: Acute Feeding**

Antibodies to egg, milk, and soya were detected in all serum samples. There was considerable variation in concentrations between subjects (10–100 fold) but values in individual volunteers changed little from experiment to experiment. No consistent pattern of change was observed in the serial, postprandial blood samples. In most subjects values altered by less than ±25%, although the percentage change was sometimes greater when the fasting values were low. The greatest range and percentage variation was with ovalbumen antibodies.

Results of immune complex assays, as determined by Clq binding, are illustrated in the Figure. After either milk (subjects 4, 5, and 6) or egg (subjects 2,
3, 4, 6, and 7) there was a substantial postprandial rise of immune complexes. The time after which the highest values were recorded varied between 30–120 minutes and usually only one very high value was obtained in each subject. In the control (protein free) experiment only one volunteer showed a postprandial response whereas none of the six subjects reacted to the soya meal. In subject 5 there was an inexplicable rise in immune complexes in the fasting sample taken before the egg experiment.

In striking contrast to the C1q results there was no evidence of increased levels of immune complexes when Raji immunoassay was used (Table 2). Only occasional values exceeded the arbitrary upper limit of normal (41 μg AHG), the highest value being 59 μg AHG.

There were no significant changes in total serum cholesterol concentrations. Postprandial increases in triglycerides were most marked after soya protein and least evident after casein.

**EXPERIMENT 2: CHRONIC FEEDING**

**Dietary data** In the soya experiment there was a substantial rise in total energy intake over the six week experimental period (11.0±0.6 SEM vs 10.1±0.7 MJ/day). Protein, fat, and carbohydrate were all increased but there was no significant change in the distribution of energy between the different nutrients. Soya consumption was negligible (0-5%) at the start of the experiment but six weeks later accounted for 22.7±2.3% of total protein.

At the start of the milk experiment volunteers and controls were eating between 12 and 48 g of milk protein per day (mean 22.4 g). With a minimum intake of 60 g milk protein during the experiment volunteers increased their consumption approximately three fold. Some subjects complained of

![Figure](image)

*Figure* Circulating immune complex concentrations by C1q binding in acute feeding experiments. Bars refer to values in individual subjects at specific times after drinking test meal. Upper limit of normal taken as 5% (horizontal broken line). For clarity entirely negative results of soya experiment are omitted.

<table>
<thead>
<tr>
<th>Subject</th>
<th>Control</th>
<th>Milk</th>
<th>Egg</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
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<td></td>
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<tr>
<td>7</td>
<td></td>
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<td></td>
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<td>8</td>
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</tbody>
</table>

Table 2 Acute experiments. Circulating immune complexes by Raji cell immune assay after a 5.5 MJ test meal

<table>
<thead>
<tr>
<th>Test meal</th>
<th>Time after test meal (min)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Control</td>
<td></td>
</tr>
<tr>
<td>(no protein)</td>
<td>13±2</td>
</tr>
<tr>
<td>Milk</td>
<td>19±7</td>
</tr>
<tr>
<td>Egg</td>
<td>13±5</td>
</tr>
<tr>
<td>Soya</td>
<td>17±4</td>
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</table>

Results expressed as means ± SEM in μg AHG equivalents per ml serum.
flatulence in the soya study and most taking milk reported a loss of appetite.

**Immunological assays**

The results of the antibody assays are summarised in Table 3. Using a paired t test no changes were observed in the mean levels of any class of antibody to either soya or milk protein. In six individuals in the soya experiment and two in the milk study a substantial increase in one or more classes of antibody was recorded. Conversely, a reduction was noted in an almost exactly equal number. The relative proportions of the various classes of serum antibodies to both milk and soya protein was similar to the distribution in normal serum. There were no appreciable changes in immune complex concentrations in either experiment.

**Cholesterol and triglycerides**

A significant increase in both total and HDL-cholesterol and triglycerides occurred during the soya experiment. There was a positive correlation between the initial and final values of plasma total cholesterol ($r=+0.56$, $p<0.05$). In the milk study there was a slight but significant fall in both total and HDL cholesterol in the test group but no changes were observed in controls. In both groups there was a significant correlation between initial and final cholesterol values (test $r=0.9$, $p<0.01$; control $r=0.95$, $p<0.001$). Triglyceride values showed no important changes in the milk feeding experiment.

**Discussion**

The object of these experiments was to study variations in food antigen specific antibodies and circulating immune complexes after both acute and chronic alterations in dietary protein. Although food antibodies were detected in all subjects these were not influenced by a three to six week milk or soya rich diet. A substantial but transient rise in immune complexes was observed in most volunteers after a meal containing 66 g of milk or egg protein. No such changes were found with soya or with a single exception the control protein free preparation.

There is no satisfactory explanation as to why healthy adults have such low concentrations of circulating food antibodies despite a continuous low grade exposure to dietary antigens from the gastrointestinal tract. An efficient mechanism must exist to maintain this form of tolerance or hyporesponsiveness, as shown by our failure to alter antibody concentrations in the chronic feeding experiments. There is good clinical and experimental evidence that the liver plays a central role in the phagocytosis of both antigenic material and immune complexes arriving via the portal venous system. Increased concentrations of bacterial and dietary antibodies can be detected in patients with advanced liver disease and hyporesponsiveness to orally administered antigens is diminished in experimental animals with hepatic cirrhosis. Cell transfer studies in this animal model suggest that T as opposed to B lymphocytes mediate the hyporesponsiveness to gastrointestinal antigens, and indeed other groups have implicated suppressor T cells in Peyer’s patches of the small intestinal mucosa. In contrast André and his colleagues found that tolerance produced by gastrointestinal challenge could be transferred by a cell free preparation rich in complexes of antigen and IgA. Animal experiments suggest that complexes containing more than two molecules of antigen and antibody are rapidly taken up by the liver and spleen. It may well be that after a milk or egg rich meal complexes of a variety of molecular sizes form but only the smaller evade phagocytosis for any period of time. Experiments in our laboratory have shown that Clq binding and Raji immunoassay both detect immune complexes greater than c $1 \times 10^6$ Daltons molecular weight, but that only Clq measures complexes smaller than this. In this context it is important that in the acute feeding experiments described above only Clq binding was positive, suggesting that postprandial circulating complexes are of relatively small molecular size.

Three previous studies have investigated the appearance of immune complexes in the circulation after milk drinking. In two of these relatively small amounts of milk were used (100 and 250 ml; 6-6 and 16-5 g protein) and complexes were detected only in patients with IgA deficiency. There was no measurable response in subjects with systemic lupus erythematosus or normal controls. In contrast Paganelli et al used much larger amounts of milk.

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**Table 3** Experiment 2: food antibody concentrations in chronic feeding studies*

<table>
<thead>
<tr>
<th></th>
<th>IgG</th>
<th>IgA</th>
<th>IgM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soya</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>63±6</td>
<td>17±1</td>
<td>26±3</td>
</tr>
<tr>
<td>Day 42</td>
<td>60±6</td>
<td>17±1</td>
<td>23±3</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>volunteers</td>
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<td></td>
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<tr>
<td>Day 0</td>
<td>2.85±0.43</td>
<td>0.79±0.16</td>
<td>0.48±0.05</td>
</tr>
<tr>
<td>Day 21</td>
<td>3.35±0.57</td>
<td>0.73±0.15</td>
<td>0.49±0.07</td>
</tr>
<tr>
<td>Milk</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>controls</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day 0</td>
<td>2.55±0.69</td>
<td>0.84±0.30</td>
<td>0.39±0.07</td>
</tr>
<tr>
<td>Day 21</td>
<td>2.56±0.71</td>
<td>0.84±0.39</td>
<td>0.37±0.07</td>
</tr>
</tbody>
</table>

* All values expressed as means ± SEM in arbitrary units. Milk and soya antibodies not quantitatively comparable.
Acute and chronic immunological response to dietary antigen

(1200 ml; 80 g protein) and found complexes by two separate techniques, including an antigen specific assay.

These results, and the findings of our own study indicate that healthy adults must consume relatively large amounts of protein before immune complexes can be measured in the circulation and that Clq binding is more likely to detect these complexes than Raji immunoassay.

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References

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