Serological diagnosis of gluten sensitive enteropathy

David J Unsworth

**Introduction**

Gluten sensitive enteropathy is the hallmark of coeliac disease but is also seen in dermatitis herpetiformis. Diagnosis is important because untreated cases are at increased risk of gut malignancy, often gastrointestinal T cell lymphomas. Gluten sensitive enteropathy is of interest because of the strong association with connective tissue autoantibodies, including the R1-anti-reticulin antibody (R1-ARA), anti-endomysial antibody (AEA) and anti-jejunal antibody (AJA) in the chronological order in which they were reported (1971, 1984 and 1986, respectively). The inter-relation between these autoantibodies has caused confusion.

The available evidence from absorption studies suggests that AEA and AJA are one and the same, simply giving different appearances when tested on different tissue substrates, such as monkey oesophagus and human foetal jejunum, respectively. Ultrastructural studies add further support. As to whether R1-ARA and AEA (and therefore AJA) are the same, they certainly show many common characteristics, but the common finding of R1-ARA negative, AEA positive coeliac disease cases is not entirely explained. Absorption studies by Hallstrom were not conclusive. Ferreira et al felt that “AEA was similar but not identical to R1-ARA”. The author’s belief is that they are the same and that the rat tissue used to detect R1-ARA/AEA is simply a less sensitive substrate than monkey oesophagus. Recently, there has been interest in the use of human umbilical cord as an alternative to monkey oesophagus for detecting these enteropathy associated connective tissue autoantibodies (see discussion under future trends).

These autoantibodies are unique in that they are predictably invoked in susceptible patients (coeliac disease) by an environmental agent, namely gluten, disappearing within several months of a strict gluten-free diet and reappearing after gluten challenge. However, in contrast to anti-wheat protein antibodies (including anti-gliadin), and other simple food antibodies, the R1-ARA is not seen in cases of enteropathy due to some other cause (for example, cow’s milk enteropathy, Giardia infection). Hence, these connective tissue autoantibodies are not generated non-specifically following any form of small bowel damage, but instead arise by unknown mechanisms only during the development of gluten sensitive enteropathy. But, the fact that gluten sensitive enteropathy has been reported in a patient with hypogammaglobulinaemia suggests that these autoantibodies are not crucial to its pathogenesis. They are, none the less, very useful diagnostic tools by virtue of their high specificity for untreated gluten sensitive enteropathy (table 1).

**Figure 1** The protein subfractions derived from wheat gluten. Note that all the gliadins, and probably the glutelins, are capable of inducing gluten sensitive enteropathy in susceptible individuals.
Serological diagnosis of gluten sensitive enteropathy

Table 1  For reference 13, the AGA results quoted were based on the ELISA method. Reference 8 included data on 81 rheumatological and other miscellaneous disease controls as well as 47 healthy subjects, in addition to the 31 gut connective tissue (GT) disease controls quoted. Very few of the controls in that study had small bowel biopsy specimens taken. Data from reference 5 were derived by the author

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CD = coeliac disease; ND = not done; PPV = positive predictive value; NPV = negative predictive value.

AVAILABLE SEROLOGICAL TESTS

These can be divided into two basic types. Firstly, antibodies against wheat gluten extracts, and, secondly, tests for gluten inducible connective tissue reactive autoantibodies (R1-ARA, AEA, AJA, etc). Figure 1 shows how gluten is derived from wheat flour and the gliadin fraction (the most toxic fraction) is prepared. Note that ethanolic cereal extracts are the prolamine extracts. Wheat prolamines are called gliadins. Barley prolamines by contrast are called hordeins.

WHICH OF THE COMMONLY USED TESTS IS THE BEST?

The AEA test is generally accepted to show the best positive and negative predictive values (table 1). However, because it uses sections of monkey oesophagus as substrate, it is expensive. Also, it occasionally comes up with the wrong result! In the author's experience, a combination of tests, beginning with a screen of high sensitivity followed by a confirmatory test with high specificity leads to reliable results. A suggested algorithm for processing routine serum samples is shown in fig 2. IgA anti-gliadin antibody (IgA AGA) as an initial screen gives a negative predictive value of around 95% in adults, but seems to be less sensitive in children (table 1). Fortunately, the IgG AGA is a very sensitive marker of gluten sensitive enteropathy in children. We therefore combine IgA and IgG AGA to form our initial screen. Also, inclusion of IgG AGA in the screen will hopefully help to detect IgA deficient cases. In practice, an initial AGA screen will miss very few cases of untreated coeliac disease. By this approach, no patient is reported as serologically likely to have active gluten sensitive enteropathy without the serum having been through at least two tests, one wheat protein based, the other autoantibody based.

Other centres use routines which differ from that suggested in fig 2. Some—for example, would advocate using the best overall test (IgA anti-endomysial) and check negative patients for IgA deficiency. Alternatively, all patients could be run through all the available tests, but this is clearly more costly. The decision partly relates to the degree of clinical suspicion. Clearly, negative initial tests in someone thought highly likely to have coeliac disease need to be backed up by further tests. Whatever process is adopted, it needs to be validated for the local population. We checked 215 of our consecutive AGA (IgA and IgG) negative patients to discover that two were IgA anti-endomysial positive (neither yet biopsied for coeliac disease).

DO WE STILL NEED TO PERFORM A CONFIRMATORY GUT BIOPSY IF THE SEROLOGY IS SO RELIABLE?

There is no doubt that modern serology has become very reliable as a means of helping select cases of possible coeliac disease for confirmatory biopsy. But serology can be misleading and there are good reasons for retaining small bowel biopsy as the diagnostic "gold standard".14 The classic pitfall with the IgA based autoantibody tests is false negative results in IgA deficient individuals (although, in practice, this rarely causes diagnostic...
difficulties in routine serological screening). Also, biopsy of seropositive patients has permitted the recognition of unusual clinical subgroups, including those with latent coeliac disease,\(^\text{15,16}\) defined as seropositivity in the face of a normal small bowel biopsy specimen but predictive of later development of enteropathy on follow up (although several years may elapse before enteropathy develops).

Finally, it should be noted that occasionally small bowel biopsy findings may be misleading\(^7\) and that “all that flattens is not sprue”. The sensible approach is to combine serology and endoscopic biopsy to ensure the most reliable diagnosis.

ANTI-GLIADIN ANTIBODY (AGA) ELISA TEST

The most popular approach is to use a solid phase ELISA assay. Crude gliadin is commercially available (Sigma, Poole, Dorset, UK). Most commercial and home made preparations will be contaminated with glutenins and other non-gliadin wheat proteins which can only be removed after complex purification steps. Fortunately, this is not necessary. There is no convincing evidence that highly purified wheat preparations such as pure alpha gliadin improves disease specificity of AGA testing.\(^8\)

In the context of using AGA tests as an inexpensive initial screen, use of crude antigen preparations is probably desirable and is certainly effective (all AGA results quoted in table 1 used crude gliadin as antigen).

ELISA METHOD

Gliadin is notoriously difficult to dissolve in saline and water. For this reason, the author uses gliadin dissolved in 70% ethanol/distilled water (final concentration of 50 mg/ml) to coat Dynatech Microelisa (Billingshurst, UK) plates at 4°C overnight as described originally in 1981\(^\text{16,17}\). 100 µl volumes are used to coat plates and in subsequent steps. The author recommends that every third row is coated with 70% ethanol/distilled water lacking gliadin to serve as a non-specific “background binding” control. Serum samples are tested in duplicate. Coated plates are washed with phosphate buffered saline (PBS) (Clin-Tech, Clacton on sea, UK) with the addition of Tween 20 (BDH, Poole, UK.) to 0.05% by volume. Coated plates can now be drained and stored in airtight plastic bags at \(-20°C\) for six months. All dilutions and washes from this point on are in PBS-Tween 20 at room temperature. Serum diluted 1 in 100 is incubated for two hours before washing (three times on an automatic ELISA plate washer). Isotype specific (IgA and IgG but no gain using IgM) enzyme conjugated anti-human immunoglobulin reagents at appropriate dilutions (depends on reagent source; determine by checkerboard titration) are incubated for two hours. We use horse radish peroxidase conjugated reagents from Dako (High Wycombe, UK) for which the working dilution is of the order 1 in 10 000. After washing, the substrate (below) is prepared fresh in a light shielded bottle and after a stopclock timed five minute incubation, the reaction is stopped with 25 µl concentrated (4 M) sulphuric acid (BDH). The optical density is read at 492 nm. A commercially available positive control serum (Sigma) can be used to establish a standard curve. A local blood donor population (at least 100 serum samples) is useful in establishing the normal levels of both IgG and IgA AGA in the local population. Values exceeding the median plus two standard deviations are considered positive.

Substrate

Always prepare fresh substrate. A single tablet (35 mg weight) of ortho-phenylene-diamine (caution — TOXIC — gloves/fume cupboard) is

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Patient with possible coeliac disease

Test for both IgG and IgA anti-gliadin as initial serological screen

IgG and/or IgA positive move to IgA autoantibody test

IgA AEA negative excludes current enteropathy, unless IgA deficient

Checking for IgA deficiency unrewarding except in high titre IgG AGA positives

Both negative coeliac disease very unlikely

IgA AEA as well as AGA positive points to active enteropathy

Recommend referral for small bowel confirmatory biopsy

? Unexpected R1-ARA (anti-reticulin) from autoantibody screens
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Figure 2 Proposed algorithm for the combined use of serology and small bowel biopsy in the diagnosis of coeliac disease.
dissolved in 100 ml buffer (50 ml distilled water, 25.7 ml 0.2M NaHPO₃, and 24.3 ml 0.1 M citric acid); 20 μl hydrogen peroxide (30% by volume) is added in the final step, and after mixing, use within a few minutes.

CLINICAL SIGNIFICANCE OF A POSITIVE AGA RESULT

Serum positive for AGA will also tend to show positivity for other food antibodies such as lactoglobulin and ovalbumin. This observation, and the fact that AGA positivity in dermatitis herpetiformis is related to the degree of small intestinal mucosal damage, suggests that AGA and other food antibodies detectable in serum reflect a non-specific sensitisation to food antigens secondary to mucosal damage, although these antibodies are often seen in patients with macroscopically normal small bowel biopsy specimens. There is general agreement that IgG AGA lack acceptable disease specificity (table 1). Some authors regard IgA AGA as useful in their own right as reliable markers of active coeliac disease. This is disputed by the author and others. IgA nephropathy is just one example of their lack of specificity for gluten enteropathy. It is, however, generally agreed that IgA AGA are more likely than IgG AGA to be associated with coeliac disease. Very few cases of untreated coeliac disease will show both IgA and IgG AGA in the normal range (table 1).

IgA DEFICIENT COELIAC PATIENTS

IgA deficient patients (said to be 1 in 50 cases of coeliac disease) are presumed to produce an IgG AGA response. Hence, AGA provides a useful initial screen and looking for IgG AGA should help to identify IgA deficient cases. Typical cases of coeliac disease will show high titres of both isotypes. The author’s policy is to select all positive results, irrespective of isotype and titre, and to retest the serum samples for IgA AEA. High titre positivity for IgA and IgG AGA as well as a positive IgA AEA test (see below) in the author’s experience (children and adults) always turns out to be associated with gluten enteropathy on small bowel biopsy. We know from other work that a normal biopsy specimen in these dual positives may relate to latency, so follow up is advised.

Perhaps all IgG AGA positive patients who do not show IgA AGA and are negative for IgA AEA should be checked for IgA deficiency. The author’s experience, however, suggests that this is largely a fruitless occupation, and an acceptable compromise is to look for IgA deficiency only in cases with high titre (at least two times the optical density reading for the upper limit of normal), isolated IgG AGA. Most cases of isolated IgG AGA positivity will not be IgA deficient nor will they have coeliac disease. However, the finding of high titre IgG AGA with IgA deficiency merits referral for small bowel biopsy.

IgA ANTI-ENDOMYSIAL ANTIBODY (AEA) TEST

This was first described by Chorzelski et al in 1984. Frozen sections of monkey oesophagus are used as substrate and are commercially available (The Binding Site, Birmingham, UK).

Home made slides should use the lower third of oesophagus obtained from Macaque monkeys. Positive control serum samples are commercially available (The Binding Site). Serum samples are screened at dilutions of 1 in 5 and 1 in 10 in PBS. Each run should include a positive control serum (available from The Binding Site). The author has seen some commercial slides which lack the lamina propria endomysium. Inclusion of a positive control serum will alert the laboratory worker to this possible pitfall. 100 μl volumes are applied to the sections which ideally are mounted on multisport slides. Incubation is for 30 minutes, followed by a 30 minute wash in a PBS slide bath equipped with a small magnetic stirrer. A fluorescein isothiocyanate (FITC) conjugated anti-human IgA isotype specific reagent (we use Dako FITC labelled anti-human IgA) is next applied at an appropriate dilution (determine by checkerboard titration—for Dako reagents, this is of the order of 1 in 50 to 1 in 100) for 30 minutes. After a final wash (30 minutes), slides are mounted in 10% glycerol/90% PBS and viewed under indirect ultraviolet illumination (Labophot 2; Nikon) at both low (×250) and high (×400) power.

Positive serum samples (fig 3) react with the connective tissue bundles which surround the smooth muscle bundles in the lamina propria, to give a lacework pattern around unstained smooth muscle cells. Sometimes, IgA anti-smooth muscle antibody is seen in the absence of AEA, and this gives almost a “negative image” of AEA (fig 4). Reactivity around the
muscle bundles in the lamina propria does not constitute a positive AEA result unless the characteristic lacework pattern is seen. Coexistence of IgA AEA and IgA anti-smooth muscle occasionally causes confusion, but in these cases, an R1-ARA test (below) will usually resolve the issue. Titration of the AEA is not helpful as a routine.

CLINICAL UTILITY OF AEA
As table 1 clearly shows, IgA AEA are highly specific and sensitive for coeliac disease. They seem to offer the best single test currently in widespread use. However, cost is the drawback.

Figure 4  Similar to fig 3, but AEA negative. Weak IgA smooth muscle antibody reactivity is shown in the muscle bundles of the lamina propria (upper half of the picture). The epidermis is just visible (negative) at the top edge of the figure.

R1-ANTI-RETICULIN ANTIBODY
First described by Seah et al in 1971 this relatively ancient test remains, in the author’s view, useful for the reasons discussed below under “clinical utility”. This antibody is detected on frozen sections cut from a tissue block composed of rat liver, kidney, and stomach (5 μm thick)—that is, the same sort of substrate that is used widely for general autoantibody screening. When first observed a striking similarity between the tissue reactivity of the new autoantibody and the tissue distribution of “reticulin” shown by histological silver impregnation (figs 5 and 6) methods (Gomori) was noted. Hence, they were called anti-reticulin antibodies. Commercial slides with sections of frozen composite rat tissues (The Binding Site) can be used. Serum samples are screened at a dilution of 1 in 10 in PBS by the same basic immunofluorescence method described above for IgA AEA. The only difference is the substrate and the pattern of immunofluorescence seen.

The key to recognition of R1-ARA is the observation on liver of peri-vascular staining with long fibrillar extensions into the surrounding liver parenchyma (fig 7). The appearance is rather like a cross section through the base of a tree showing roots extending outwards. Elsewhere in the liver parenchyma, isolated hair-like fibrils are seen. These features on rat liver are the most reliable features of true R1-ARA. Many alternative patterns of connective tissue-like staining may be seen on liver but most often these reactivities are due to heterophile antibodies, which are usually IgG and react around liver sinusoids. A positive
result can only be recorded with confidence when the full R1-ARA pattern is seen on kidney and stomach as well as liver. On kidney, fibrillar outlining around all the kidney tubules and glomeruli is seen (fig 8). (Note similarity to silver impregnation pattern shown in fig 5.) On stomach, endomysial-like lacework in the subglandular muscle bundles below the muscularis mucosae, and fibrillar staining in between the gastric glands is seen (fig 9). For practical purposes, when R1-like staining is seen on some but not all three tissues, it is advisable to record a negative result for R1-ARA but retest the serum for AGA and AEA.

CLINICAL UTILITY OF R1-ARA
As seen in table 1, R1-ARA are highly specific for gluten sensitive enteropathy. However, this seems to be so only in experienced hands. Difficulty in recognition stems in part from the fact that there are at least five anti-reticulin staining patterns. Only the type 1 (R1-ARA) is associated with coeliac disease. The AEA test is easier to interpret and more sensitive for coeliac disease without loss of disease specificity. The author has never seen an IgA R1-ARA positive that does not also show IgA AEA positivity, though the converse is not true, supporting the notion that both antibodies are the same but monkey oesophagus a more sensitive substrate. The practical point is that coeliac disease associated connective tissue antibodies will show up in a routine autoantibody test such as R1-ARA. Thus, if a patient with coeliac disease is subjected to autoantibody testing for any reason (unexplained fatigue, anaemia, other) R1-ARA may show up, and correct interpretation and reporting can increase the incidence of coeliac disease by helping to identify atypically presenting cases with occult coeliac disease. Because autoantibody screening uses a polyspecific anti-human immunoglobulin reagent, the R1-ARA pick up is suboptimal in routine autoantibody testing. Suspected positives from an autoantibody screen should therefore be supported by testing for IgA R1-ARA, and for AGA and IgA AEA.

ANTI-JEJUNAL ANTIBODY
This test is not in common usage. Cryostat sections (unfixed) of human foetal gut or normal human jejunum are used in an IgA specific test as described. The test is no better than the AEA test and is not discussed further. Interestingly, on theoretical grounds, human umbilicus should be no better than human jejunum and therefore no better than monkey oesophagus despite claims that it is.

Figure 8 IgA R1-ARA by indirect immunofluorescence on rat kidney. Two glomeruli, top right and middle left. Reticular fibrils are stained around all tubules and glomeruli, similar to fig 5.

Figure 7 IgA R1-ARA by indirect immunofluorescence on rat liver. This (green) pattern of tissue reactivity is pathognomonic for R1-ARA. See text for full description. Note that silver impregnation gives a different, less restricted pattern of staining, especially around sinusoids.

Figure 9 IgA R1-ARA on rat stomach. Endomysial-like staining (top) is seen in the submucosa and thick reticulin fibrils are stained along the length of the gastric glands (bottom).
Future trends
At present, the connective tissue autoantibody tests show the best positive predictive values (see table 1) but rely on indirect immunofluorescence using tissue sections as substrate. Thus, interpretation has a subjective aspect and it is likely that solid phase assays using purified connective tissue (reticulin/endomysium) autoantigens will become the definitive methods. Despite initial reports that “reticulin” has been purified,23 its structure and composition remain obscure, and solid phase assays are not likely to be available for some time. Candidate autoantigens have included collagen type III and fibronectin, which both codistribute with “reticulin” in tissue sections, but absorption studies argue against these two connective tissue antigens being the autoantigens in coeliac disease.25

Human umbilical cord as substrate for coeliac associated connective tissue autoantibodies
The author has limited experience with this substrate but agrees that it shows promise. The original reports’ recommend acetone fixation of slides, but this in fact is not necessary. Blood group O umbilical cord tissue is not a mandatory requirement in an IgA based test given that isohaemagglutinins are of IgM isotype. Heterophile antibodies and the non-R1 reticulin antibodies are mainly IgG26 and are similarly not expected to interfere in an IgA based assay. The IgG anti-endomysial test not surprisingly produces more false positives and is best avoided. The world-wide experience at this time with monkey oesophagus far exceeds that with umbilical cord testing, and in the author’s view, there is a need to test many more serum samples in several different expert centres before a switch to umbilical cord substrate is recommended. Some of our initial results (based on adults attending for small bowel biopsy for possible coeliac disease (table 2) suggest that the umbilical cord test gives similar results to monkey oesophagus, but raises the as yet unsubstantiated worry that umbilical cord may yield slightly more false positive results. All IgA R1-ARA and AEA positive patients listed in table 2 had coeliac disease. One false negative and four IgA umbilical cord false positive results were noted, perhaps due to our inexperience with the new test. We are continuing to evaluate the method. Perhaps screening with an IgA umbilical cord test (inexpensive), confirming positive results on monkey oesophagus will be a robust approach in routine practice. See fig 10 for a typical positive IgA test on umbilical cord and fig 11 for the common finding of non-specific weak smooth muscle reactivity.

Table 2 Comparison of IgA autoantibody tests in adults using umbilical cord and monkey oesophagus as substrate

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Figure 10 IgA reactivity on unfixed human umbilical cord. Note connective tissue reactivity in between muscle bundles (endomysial reactivity – left) and staining of cells within Wharton’s jelly (right).

Figure 11 Negative result on umbilical cord. Note smooth muscle reactivity is often seen with non-coeliac serum samples.

Thanks to Mr Bob Lock (Department of Clinical Immunology, Southmead Hospital, Bristol) for permission to quote umbilical cord data and for figs 10 and 11. I am also grateful to Dako Ltd, High Wycombe, Bucks, UK, for agreeing to cover the costs of production of the colour photographs shown in this article.

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23 Unsworth DJ, Brown DL. Serological screening suggests that adult coeliac disease is underdiagnosed in the UK and increases the incidence by up to 12%. Gut 1994;35:61-4.
D J Unsworth

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