larger studies in which both methods are evaluated against clinical outcome are needed.

Most authorities agree that the presence of xanthochromia is the primary biochemical criterion for a diagnosis of SAH, but it may be absent, especially if the lumbar puncture was done <12 h after the event (1). In these cases, an increase in CSF oxyhemoglobin may be the only biochemical abnormality. Unfortunately, CSF oxyhemoglobin may be increased by traumatic lumbar puncture (2).

In conclusion, automated bilirubin measurement is an easy and robust test and can be used in a stat environment to screen patients for increased NBA as defined by spectrophotometric scanning. The method can easily be performed on automated chemistry analyzers, using the on-board serum bilirubin reagent, although individual laboratories should validate their own decision limits.

References


Previously published online at DOI: 10.1373/clinchem.2004.033282

Anti-Transglutaminase Antibodies and Age, Valentina Baldas,1 Tarcisio Not,1 Alberto Tommasini,1 Filippo Ansaldi,2 Sergio Demarin,2 Daniele Sblattero,2 Roberto Marzari,2 Lucio Torelli,2 Alberto Burlina,2 Claudio Tiribelli,7 and Alessandro Ventura1 (1) Clinica Pediatrica, 2 Istituto d’Igiene, 4 Dipartimento di Biologia, 3 Dipartimento di Scienze Matematiche, and 7 Centro Studi Fegato and Dipartimento di Biochimica, Biofisica, Chimica delle Macromolecole, Università di Trieste, Trieste, Italy; 3 Divisione di Neonatologia IRCCS Burlo Garofolo Trieste, Trieste, Italy; 6 Dipartimento di Pediatría, Università di Padova, Padua, Italy; * address correspondence to this author at: Clinica Pediatrica, IRCCS “Burlo Garofolo”, Via dell’Istria 65/1, 34100 Trieste, Italy; fax 39-0403785210, e-mail not@burlo.trieste.it

The tendency within the general population to produce both organ and non-organ-specific autoantibodies is well recognized (1). It is not clear whether these autoantibodies represent the subclinical spectrum of certain autoimmune diseases or whether they are one effect of aging on the autoimmune system (2,3). Celiac disease (CD) is an autoimmune disorder triggered by ingestion of gluten; it occurs mainly in individuals carrying the celiac-related HLA DQ2/8 (4). The discovery that the enzyme tissue transglutaminase (tTG) was the CD autoantigen has led to the design of various human tTG-based immunoassays to measure the specific antibodies of CD for diagnostic purposes. The widespread adoption of these assays indicates the diagnostic value of anti-human (t)TG, with its high sensitivity (98%) and specificity (95%) (5–7). Epidemiologic studies using this test have revealed that CD is one of the most common lifelong disorders, with a childhood prevalence of 1 in 90 individuals (8,9). We investigated the relationship between age and the serum concentrations of anti-human-tTG autoantibodies in apparently healthy individuals, focusing in particular on identifying the age-dependent cutoff limits for the general population.

The study was performed retrospectively on sera from 4575 individuals (2431 females and 2144 males; median age, 9 years; range, 3 days–82 years) participating in two large screening programs, one for CD in the general population and the other for metabolic diseases in newborns and children. Anti-human-tTG values of screened individuals diagnosed as having CD (46 of 4575) were excluded.

In addition, we compared antibody concentrations in the study group with the IgA and IgG antibody concentrations of 144 patients with biopsy-confirmed CD diagnosed at the Children’s Hospital “Burlo Garofolo” from January 2002 to September 2003.

The study population enrolled included both healthy individuals and untreated CD patients classified as follows: Group 1 included 145 sera from unselected newborns at the third day of life (75 females and 50 males) and 49 sera from healthy children (29 females and 20 males; mean age, 1.8 years; range, 1–5 years) obtained as part of a nutritional study.

Group 2 included 2774 sera from schoolchildren (1393 females and 1381 males; median age, 9 years; range, 5–12 years) in Trieste as part of a screening for CD (9) and 257 sera from teachers collected during the school-based screening (127 females and 130 males; median age, 35 years; range, 22–55 years).

Group 3 included 1350 serum samples collected in 1991 (797 females and 553 males; median age, 48 years; range, 12–82 years) from the population of Cormons as part of the Dionysos Study (10). Individuals were at risk factors for liver disease, such as alcohol use or hepatotropic viruses. The sera were stored at −30°C and thawed only once before use.

Group 4 included 144 sera from patients with biopsy-confirmed CD (82 females and 62 males; median age, 12 years; range, 1–68 years). Eleven patients (8 females and 3 males; median age, 10 years; range, 2–20 years) had total IgA serum deficiency (IgA <50 mg/L).
We divided the study group into eight age groups (Table 1A). The groups in the lower age ranges (3 days–20 years) covered shorter time intervals than the adult groups (21 to >70 years); this enabled us to identify more precisely the changes in antibody titer in pediatric age groups.

Serum IgA and IgG anti-human-tTG antibodies were measured by ELISA (11, 12) in plates coated with 1 g/well of highly purified human recombinant tTG. Serum samples diluted 1:100 were incubated for 1 h. The plates were washed and incubated for 1 h with either phosphatase conjugated anti-human IgA (diluted 1:3000; cat. no. A-3062; Sigma) or anti-human IgG (diluted 1:7000; cat. no. A-8542; Sigma). The immune reaction was developed by adding substrate solution (1 g/L p-nitrophenyl phosphate), and the absorbance was read at 405 nm. Results are expressed as the percentage of the positive control serum. Among the children, the sensitivity and specificity of our test were 98% and 97%, respectively, with good reproducibility for both the IgA and IgG antibodies (intra- and interassay CVs, 2.3% and 3.9%, respectively, for IgA and 3.9% and 6.5% for IgG).

Pearson correlation coefficients were calculated based on age and IgA and IgG anti-human-tTG antibodies concentrations in healthy individuals and in CD patients. Differences in antibody concentrations across age groups were tested by ANOVA with a post hoc Bonferroni–Dunn test. The data were plotted as box plots. The sensitivity and specificity of the ELISA tests were calculated at positive and negative cutoffs corresponding to the 0.5, 1, and 3 percentiles of IgA and IgG serum concentrations in CD patients.

To evaluate the effect of the storage time on the anti-human-tTG titers, we compared the antibody titers of the negative sera collected in 1991 with the antibody titers of the sera collected in 2003 from 100 randomly selected

Table 1. IgA and IgG anti-tTG titers in healthy individuals and patients with CD.

A. Mean (SD) titers* in the eight age-related study groups (healthy individuals) and the two celiac groups

<table>
<thead>
<tr>
<th>Healthy individuals</th>
<th>n</th>
<th>%</th>
<th>Mean (SD) IgA, %</th>
<th>Mean (SD) IgG, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborns</td>
<td>145</td>
<td>3.1</td>
<td>0.4 (1.2)</td>
<td>5 (5.5)</td>
</tr>
<tr>
<td>1–6 years</td>
<td>520</td>
<td>11</td>
<td>3.2 (2.9)</td>
<td>11.5 (6.8)</td>
</tr>
<tr>
<td>7–10 years</td>
<td>2247</td>
<td>47.7</td>
<td>3.1 (3)</td>
<td>10.5 (7.5)</td>
</tr>
<tr>
<td>11–20 years</td>
<td>146</td>
<td>3.1</td>
<td>3.4 (3)</td>
<td>11.6 (8.1)</td>
</tr>
<tr>
<td>21–40 years</td>
<td>537</td>
<td>11.4</td>
<td>7.2 (4.7)</td>
<td>20.3 (10.2)</td>
</tr>
<tr>
<td>41–60 years</td>
<td>546</td>
<td>11.5</td>
<td>8.5 (4.8)</td>
<td>22.3 (10.4)</td>
</tr>
<tr>
<td>61–70 years</td>
<td>293</td>
<td>6.2</td>
<td>10.5 (5.9)</td>
<td>23.4 (11.8)</td>
</tr>
<tr>
<td>&gt;70 years</td>
<td>141</td>
<td>3</td>
<td>10.9 (6.8)</td>
<td>24.9 (11.6)</td>
</tr>
<tr>
<td>CD patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>All ages</td>
<td>133</td>
<td>2.8</td>
<td>73.8 (39.1)</td>
<td>48.7 (29)</td>
</tr>
<tr>
<td>Only with IgA serum deficiency</td>
<td>11</td>
<td>0.2</td>
<td>0</td>
<td>105.5 (32.5)</td>
</tr>
</tbody>
</table>

B. Specificities* of the IgA and IgG anti-tTG antibodies in the eight age-related study groups (healthy individuals) and two groups of patients with untreated CD

<table>
<thead>
<tr>
<th>Healthy individuals</th>
<th>&lt;10%</th>
<th>&lt;12.4%</th>
<th>&lt;17%</th>
<th>Mean (SD) IgA, %</th>
<th>Mean (SD) IgG, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Newborns</td>
<td>145</td>
<td>100</td>
<td>145</td>
<td>100</td>
<td>103</td>
</tr>
<tr>
<td>1–6 years</td>
<td>497</td>
<td>95.6</td>
<td>512</td>
<td>98.5</td>
<td>69</td>
</tr>
<tr>
<td>7–10 years</td>
<td>2156</td>
<td>96</td>
<td>2208</td>
<td>98.3</td>
<td>2156</td>
</tr>
<tr>
<td>11–20 years</td>
<td>139</td>
<td>95.9</td>
<td>142</td>
<td>97.9</td>
<td>139</td>
</tr>
<tr>
<td>21–40 years</td>
<td>421</td>
<td>78.3</td>
<td>473</td>
<td>87.9</td>
<td>17</td>
</tr>
<tr>
<td>41–60 years</td>
<td>373</td>
<td>68.3</td>
<td>443</td>
<td>81.1</td>
<td>15</td>
</tr>
<tr>
<td>61–70 years</td>
<td>165</td>
<td>56.3</td>
<td>198</td>
<td>67.6</td>
<td>7</td>
</tr>
<tr>
<td>&gt;70 years</td>
<td>77</td>
<td>54.6</td>
<td>97</td>
<td>68.8</td>
<td>124</td>
</tr>
<tr>
<td>Total</td>
<td>3973</td>
<td>84.7</td>
<td>4218</td>
<td>92.2</td>
<td>4494</td>
</tr>
</tbody>
</table>

CD patients c

<table>
<thead>
<tr>
<th>&lt;5%</th>
<th>&lt;5.3%</th>
<th>&lt;9%</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Titers are expressed as percentage relative to the titer of the positive serum control.

Specificities were derived by use of three cutoffs, the 0.5, 1, and 3 percentiles of antibody titers in the CD patients.

The results shown are for the two CD patients who had false-negative results.

We divided the study group into eight age groups (Table 1A). The groups in the lower age ranges (3 days–20 years) covered shorter time intervals than the adult groups (21 to >70 years); this enabled us to identify more precisely the changes in antibody titer in pediatric age groups.

Serum IgA and IgG anti-human-tTG antibodies were measured by ELISA (11, 12) in plates coated with 1 μg/well of highly purified human recombinant tTG. Serum samples diluted 1:100 were incubated for 1 h. The plates were washed and incubated for 1 h with either phosphatase conjugated anti-human IgA (diluted 1:3000; cat. no. A-3062; Sigma) or anti-human IgG (diluted 1:7000; cat. no. A-8542; Sigma). The immune reaction was developed by adding substrate solution (1 g/L p-nitrophenyl phosphate), and the absorbance was read at 405 nm. Results are expressed as the percentage of the positive control serum. Among the children, the sensitivity and specificity of our test were 98% and 97%, respectively, with good reproducibility for both the IgA and IgG antibodies (intra- and interassay CVs, 2.3% and 3.9%, respectively, for IgA and 3.9% and 6.5% for IgG).

Pearson correlation coefficients were calculated based on age and IgA and IgG anti-human-tTG antibodies concentrations in healthy individuals and in CD patients. Differences in antibody concentrations across age groups were tested by ANOVA with a post hoc Bonferroni–Dunn test. The data were plotted as box plots. The sensitivity and specificity of the ELISA tests were calculated at positive and negative cutoffs corresponding to the 0.5, 1, and 3 percentiles of IgA and IgG serum concentrations in CD patients.

To evaluate the effect of the storage time on the anti-human-tTG titers, we compared the antibody titers of the negative sera collected in 1991 with the antibody titers of the sera collected in 2003 from 100 randomly selected
Fig. 1. Relationship between IgA and IgG anti-human tTG antibody titers in the eight healthy age groups and the patients with untreated CD. Box plots show the median, interquartile range, outliers, and extreme cases of variables. *, $P < 0.001$; o, $P < 0.01$; §, $P < 0.05$. 

Technical Briefs
individuals belonging to group 3. Sequential serum samples were compared by the Wilcoxon signed-rank test.

In the healthy population, there was a clear correlation between age and concentrations of both IgA \( (r = 0.557; P < 0.01) \) and IgG \( (r = 0.599; P < 0.01) \) anti-human-tTG antibodies, whereas we observed no correlation in patients with biopsy-confirmed CD \( [r = 0.087 (P = 0.32) \) for IgA; \( r = 0.01 (P = 0.99) \) for IgG]. The numbers of healthy individuals and CD patients in each age group and their antibody concentrations are collated in Table 1A and shown in Fig. 1. Titers of anti-human-tTG IgA and IgG were significantly lower in newborns. There was no significant difference in IgA anti-human-tTG antibodies among the 1–6, 7–10, and 11–20 year age groups; however, these groups had significantly lower antibody concentrations than the four older age groups. Indeed, IgA was sharply increased in the 21–40 age group, with a more gradual increase in the three groups >40 years (Fig. 1). IgG anti-human-tTG concentrations were also sharply increased in the 21–40 age group (Fig. 1).

The specificity of the ELISA test, calculated with cutoffs corresponding to the 0.5, 1, and 3 percentiles of anti-human-tTG IgA and IgG concentrations in sera from CD patients, is shown in Table 1B. The 0.5, 1, and 3 percentile values were 10%, 12.4%, and 17% and 5%, 5.3%, and 9% for IgA and IgG, respectively. These percentile values provided sensitivities of 99.5%, 99%, and 97%, respectively.

We investigated gender differences in concentrations of the autoantibodies among the study groups. The 2144 healthy males had a higher mean (SD) IgA anti-human-tTG concentration than the 2431 females [7.2 (3.6)% for the females vs 8.0 (3.6)% for the males; \( P < 0.01 \)] and a lower IgG anti-human-tTG concentration [20 (7.9)% for the males vs 21.0 (8.4)% for the females; \( P = 0.01 \)]. Among the untreated CD patients, there was no significant gender difference either for anti-human-tTG IgA [73.9 (36.7)% for males vs 73.7 (41.1)% for females; \( P = 0.98 \)] or IgG [49.6 (32.6)% for males vs 47.9 (26.0)% for females; \( P = 0.74 \)].

The small difference observed between healthy males and healthy females had little effect on specificity: with IgA, for example, the specificity observed in females in the 7–10 year age group was 96%, 98%, and 100%, respectively, at the 0.5, 1, and 3 percentile cutoff values. These findings are compatible with the data describing the entire 7–10 year population reported in Table 1B; the findings were the same for the other groups. No differences among the autoantibody concentrations were noted between the negative sera collected in 1991 and the sera collected in 2003 from the same 100 healthy individuals [for the 1991 samples, median IgA = 6.5% (range, 0–16%); median IgG = 17.5% (7–36%); for the 2003 samples, median IgA = 5% (0–14%); median IgG = 15% (2–35%); \( P = 0.9 \) for IgA and 0.7 for IgG].

This is the first study to demonstrate an age-dependent increase in both anti-transglutaminase IgA and IgG titers in the general population. In particular, there was a striking increase in both immunoglobulin isotypes in the 21–40 age range; this increase plateaued among individuals older than 60 years. The first practical consequence of this age-related fluctuation in anti-transglutaminase autoantibodies was a change in IgA and IgG cutoff limits among the age groups studied. This enabled us to distinguish the pediatric population from the adult population with more precision and allowed us to potentially disregard unexpected positive sera with minor increases in anti-tTG titer, as reported recently in a nonceliac adult population (13, 14).

Use of the new cutoffs derived from the 0.5 and 1 percentiles of IgA anti-human-tTG titers for the CD patients among the individuals <20 years of age gave specificities as high as >95.6% and >97.9%, respectively, whereas use the third percentile as cutoff in the age groups >21 years gave specificities between 96.3% and 87%, depending on the group. These new age-dependent cutoffs should greatly improve the specificity of the IgA anti-transglutaminase antibody assays, with accompanying improvements in negative predictive values. This enhanced specificity should not affect sensitivity values because all of the age-related cutoff limits were below the third percentile of the IgA values of our untreated celiac patients at diagnosis.

With IgG anti-transglutaminase antibody, our new age-dependent cutoff limits improve specificity but seem to lower the sensitivity of the test. These limitations of IgG for diagnostic purposes are already well known from pediatric clinical experiences, in which IgG demonstrated low sensitivity (54–89%) with fairly good specificity (93–96%) (6, 12, 15). The clinical usefulness of IgG seems to be limited to identifying those CD patients with total serum IgA deficiency (2.6% of CD patients) (16) and those with serum IgA concentrations within the reference interval but who produce only specific IgG (17). In our experience, both of these groups have very high concentrations of specific IgG autoantibodies. Because case-finding screening programs can now be set up for children (18) and adults (19) at risk for undiagnosed CD, the accurate determination of age-related cutoff limits is crucial (20). With this in mind (and to avoid false-positive test results, which may lead to unnecessary intestinal biopsies), highly specific tests are essential.

Finally, although the difference in antibody titers in males and females reached statistical difference, this had no effect on specificity values for the various healthy age groups, which were identical to those in the population not divided by gender.

In conclusion, there is a clear age-related increase in antibody titers to tTG autoantibodies. Consequently, for both diagnostic purposes and eventual mass screening programs, cutoff points for anti-human tTG should be calculated on the basis of age group.

This study was supported by research grants from IRCCS “Burlo Garofolo” (Grant RF174/02), from MIUR cofin 2001063713_001, and from Fondo studi fegato-ONLUS.
References


DOI: 10.1373/clinchem.2004.036012