do not advocate the routine use of the LDDST-CRH test for the detection of recurrent hypercortisolism in patients who have undergone pituitary surgery for Cushing disease.

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Hemolysis Interferes with the Detection of Anti–Tissue Transglutaminase Antibodies in Celiac Disease

To the Editor:

Detection of anti–tissue transglutaminase (tTG)[1] antibodies by the use of human recombinant or erythrocyte tTG-IgA–based ELISA assays is 1 of the preferred tests for diagnosing celiac disease (CD) *(1)*. However, studies comparing different tTG kits have revealed variable sensitivities, raising concern in clinical practice *(2)*. Erythrocytes (RBC) contain tTG *(3)*. In patients, upon hemolysis, the endogenous RBC-tTG released may immunoprecipitate with anti-tTG antibodies and interfere with their detection. Newer anti–deamidated gliadin peptide (DGP)-IgA assays, however, should not be affected *(4)*. To assess the effect of hemolysis on tTG-IgA titers, we used stored sera from 9 patients with biopsy-confirmed, active CD who gave informed consent for study participation. Samples were divided into 3 groups (n = 3, in each) according to the tTG-IgA concentrations after thawing [high titer (>185 U), intermediate titer (100–140 U), and borderline titer (20–50 U)]. A whole-blood sample from 1 tTG/DGP-seronegative patient, which contained 149 g/L of hemoglobin (HGB), was hemolyzed by freezing and thawing until >90% of cells were lysed, then serially diluted (1:2, 1:5, 1:10, 1:50, 1:100, 1:500) in PBS to obtain HGB concentrations of 67.1, 26.8, 13.4, 2.7, 1.3, and 0.27 g/L, respectively, and finally, added to each sample at a 1:1 ratio. For the tTG sequestration experiment, human recombinant tTG from Diarect AG was added for a final concentration of 0.04, 0.02, 0.01, and 0.002 g/L. Undiluted serum was used as the initial titer reference, and serum diluted 1:2 in PBS as the control. Antibody titers were quantified by using 2 ELISA kits: QUANTA Lite™ h-tTG IgA (human erythrocyte tTG-IgA based) and Gliadin II (DGP-IgA based) from INOVA Diagnostics, Inc. Assays were performed blinded following manufacturer's instructions. Differences between groups were evaluated by using the Mann–Whitney *U*-test, with P values <0.05 considered significant.

We found that addition of hemolyzed blood (HB) to sera of

[1] Nonstandard abbreviations: tTG, tissue transglutaminase; CD, celiac disease; RBC, erythrocytes; DGP, deamidated gliadin peptides; HGB, hemoglobin; HB, hemolyzed blood.

patients with active CD led to reduction of anti-tTG, but not anti-DGP antibodies in all groups, with significant reduction in the intermediate- and borderline-titer groups. Overall mean titer losses of anti-tTG vs anti-DGP antibodies in the high-titer, intermediate-titer, and borderline-titer groups were 36% vs 13%, 45% vs 3% ($P = 0.026$), and 51% vs 2% ($P = 0.0022$), respectively. We also found that addition of increasing concentrations of HGB reduced the titers of anti-tTG, but not of anti-DGP, in a concentration-dependent manner and induced negative anti-tTG results in samples with low tTG antibody concentrations. The anti-tTG titer decreased 2%–65%, 1%–81%, and 16%–74% (for HGB concentrations of 0.3–67.1 g/L) in the high-, intermediate-, and borderline-titer groups, respectively, compared with a decrease of 10%–16%, 4%–8%, and 7%–3% in the anti-DGP titer. In all groups, tTG titer reduction was greater with higher concentrations of HB/HGB and gradually recovered when the red tinge started to disappear at approximately 13 g/L of HGB, until complete visual disappearance (approximately 0.3g/L HGB). In the intermediate- and borderline-titer groups, titer reduction induced false-negative results (<20 U) with the anti-tTG but not anti-DGP assays for HGB concentrations ≥13 or ≥0.3 g/L, respectively (Fig. 1). A third finding was that addition of increasing concentrations of exogenous tTG (recombinant human tTG) to intermediate-titer sera caused a significant reduction in anti-tTG assay titers similar to that seen with HGB (range, 32%–82%; mean, 69%) compared with that of anti-DGP titers (mean, 18%; range, 1%–38%; $P = 0.0159$).

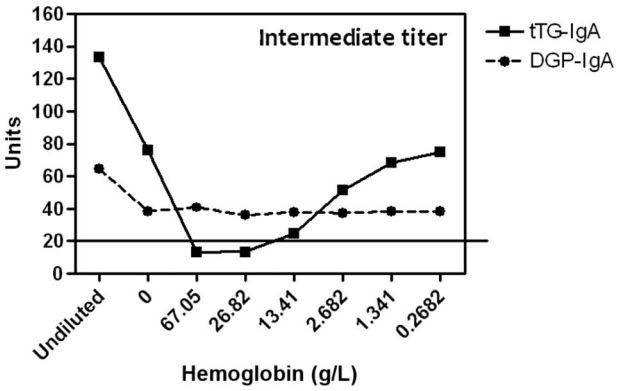

Fig. 1. Change in tTG and DGP antibody titers after incubation with hemolyzed blood.

Serum samples from 9 patients with active CD, with 3 different concentrations of tTG and DGP-IgA antibodies (high, intermediate, and borderline), were incubated with decreasing concentrations of HB/HGB from a seronegative patient and then tested for both tTG and DGP-IgA antibody concentrations by ELISA. Data for only the intermediate-titer group are shown. Assay titers, as arbitrary units, are plotted on the vertical axis; and HGB concentrations, correspondent to the serial dilution of hemolyzed blood (1:2, 1:5, 1:10, 1:50, 1:100, 1:500), on the horizontal axis. Values represent the mean antibody titer of 3 patients for control (undiluted and no HB/HGB) and for hemolyzed samples (HGB range 67.1–0.3g/L). Horizontal line= manufacturer's cutoff value (20U).

Hemolysis, visually detected by a red tinge in the serum/plasma, is commonly seen in clinical practice. Usually caused by improper transportation or venipuncture and occasionally by medical conditions, hemolysis is one of the most common causes of specimen rejection by laboratories, and requires the specimen to be redrawn *(5)*. Visible hemolysis begins at around 0.5 g/L of HGB and is evident above 1.3 g/L *(5)*. Our study demonstrated that hemolysis interferes with the detection of anti-tTG antibodies, leading to false-negative anti–tTG-IgA results when the sample is visibly hemolyzed. This interference is most significant when gross hemolysis is present (approximately 2.5 g/L). The anti–DGP-IgA assay, however, was unaffected by hemolysis. This phenomenon was not limited to the INOVA human-RBC–based tTG assay but was also seen with recombinant-tTG–based assays (The Binding Site; Eu-tTG IgA Umana, Eurospital; and EliA Celikey, Phadia) (data not shown). Similar concentration-dependent titer reduction by addition of exogenous recombinant human tTG suggests that sequestration of anti-tTG antibodies by endogenous RBC-tTG is the mechanism responsible. These findings could explain some false-negative results seen in CD diagnosis when tTG-IgA assays are used. Clinicians and laboratories should strive to avoid hemolysis. If it is detected, however, physicians should be notified and blood samples redrawn. If these actions are not possible, samples should be quantified for anti-DGP antibodies.

In conclusion, hemolysis can interfere with diagnosis of CD by sequestration of anti-tTG antibodies, especially in patients with low titers of tTG-IgA. Consequently, anti-tTG should not be determined in samples with visible hemolysis. Anti-DGP serological tests are

not affected by hemolysis and are suggested when hemolysis is suspected.

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High Glucose Upregulates C-Reactive Protein Synthesis in Macrophages

To the Editor:

C-reactive protein (CRP)[1] released from hepatocytes during the acute-phase response is a diagnostically sensitive systemic marker for inflammation; CRP also demonstrates substantial proinflammatory effects *(1)*. Although CRP might have an important role in the pathogenesis and prediction of coronary heart diseases *(2)*, the factors influencing its concentration are not yet well understood. Recent histologic investigations have demonstrated that CRP is present in the human arterial intima of atherosclerotic lesions *(3)* and is located in macrophages of the arterial plaque. In addition, macrophages have been shown to produce CRP mRNA *(4)*.

Diabetes mellitus is associated with premature and accelerated atherosclerosis *(5)*, and hyperglycemia accelerates atherosclerosis by inducing vascular dysfunction and an increased inflammatory burden. Our goal was to analyze the possible modulation of CRP production in macrophages after their exposure to high glucose concentrations.

J-774A.1 macrophage-like cells (ATCC) were plated in DMEM with 100 000 U/L penicillin, 100 mg/L streptomycin, and 2 mmol/L glutamine (P/S/G), and used within 7 days. THP-1 human monocyte cells were maintained in RPMI-1640 medium with P/S/G, and macrophage induction was achieved by incubation with phorbol myristate acetate. Cells were incubated with either 5–40 mmol/L glucose for 18 h or with 22 nmol/L mannitol (an osmotic control that did not appreciably affect CRP cellular production).

We extracted cellular RNA with MasterPure™ RNA Purification Kit (Epicentre Biotechnologies) and prepared cDNA with the Verso™ cDNA Kit (Thermo Scientific) according the manufacturers' instructions. CRP production was measured by quantitative real-time PCR by means of Rotor-Gene 6000 (Corbett Life Science/Qiagen) amplification with ABsolute Blue QPCR ROX Mix (Thermo Scientific) and primers and probes for the *CRP*[2] (C-reactive protein,

[1] Nonstandard abbreviations: CRP, C-reactive protein; P/S/G, 100 000 U/L penicillin, 100 mg/L streptomycin, and 2 mmol/L glutamine.

[2] Human genes: *CRP*, C-reactive protein, pentraxin-related; *ACTB*, actin, beta.