

## Anti-Gal Titers in Healthy Adults and Inflammatory Bowel Disease Patients

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### ABSTRACT

**Introduction.** ALPHA-GAL is a glycoconjugate present on cell membranes of mammals and bacteria but not humans who display anti-Gal antibodies (AB) in high titers provoked by the commensal gut flora. In the present study, we sought to determine the longitudinal course of alpha-Gal specific AB titers of all isotypes over 8 weeks among healthy adult subjects. Furthermore, we hypothesized that inflammatory bowel disease (IBD) patients display increased anti-Gal titers.

**Materials and methods.** We drew serum from healthy probands ( $n = 20$ ) weekly for 8 weeks and obtained plasma samples of from patients suffering from Crohn's disease ( $n = 20$ ) and ulcerative colitis ( $n = 20$ ). We measured anti-Gal ABs of all isotypes and total immunoglobulin (Ig) content using an enzyme-linked immunosorbent assay technique. For statistical evaluation of the longitudinal titers, we calculated confidence intervals for the slopes of a random intercept model, comparing variances between and within the probands. For group comparisons, we performed paired student *t*-tests and Pearson correlations.

**Results.** Alpha-Gal specific IgG, IgM, IgD, and IgA titers remained unvaried within a narrow range upon longitudinal observation. Most probands did not display alpha-Gal specific IgE ABs. Crohn's disease patients showed highly increased alpha-Gal-specific IgA titers compared with control subjects ( $P < .01$ ).

**Conclusion.** Apart from IgE, alpha-Gal-specific ABs of all isotypes remained constant over longer time periods in healthy subjects. Thus, significant titer changes actually represent increased antigen exposure and a specific anti-alpha-Gal response. Crohn's disease patients display increased anti-Gal IgA titers compared with healthy controls, which reflects a chronically impaired mucosal gut barrier in this patient cohort.

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**THE ALPHA-GAL EPITOPE** (Gal $\alpha$ 1.3-Gal $\beta$ 1-4GlcNAc-R) and corresponding human anti-Gal antibodies (ABs) described by Galili et al<sup>1</sup> are widely known to be the major cause of hyperacute rejection in pig-to-primate organ transplantation, leading to the successful production of alpha-1.3 GT knockout (KO) pigs.<sup>2</sup>

Our group showed that alpha-Gal residues are present in commercially available bioprosthetic heart valves, provoking specific immunoglobulin (Ig)M and IgG anti-Gal responses after implantation.<sup>3,4</sup> Lila et al showed greater calcification potential of Gal-positive tissue, which raised the question of alpha-1.3GT KO pigs as a possible source

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for valve tissue.<sup>5</sup> This attempt seems to be promising, although methodological questions can be raised as discussed elsewhere.<sup>6</sup>

Up to now, every Ig isotype directed against alpha-Gal has been investigated in the serum. IgA was already detected by Koren et al in 1993.<sup>7</sup> Anti-Gal IgE was identified to be responsible for anaphylactic reactions after consumption of red meat in some atopic patients.<sup>8</sup> Even the orphan IgD isotype seems to be elevated in atherosclerosis patients.<sup>9</sup> The origin of those high anti-Gal titers is believed to be a response to a constant antigenic stimulation by Gal-containing commensal gut bacteria. This hypothesis was recently questioned by Dahl et al, since Gal KO mice do not spontaneously develop anti-Gal ABs, although the gut flora is highly Gal-positive.<sup>10</sup>

We considered the alpha-Gal/anti-Gal system as an ideal model to evaluate immunologic interactions between humans and their environment with regard to host defense, tolerance, and autoimmunity, especially concerning immunologic homeostasis in the gastrointestinal tract. In the present study, we investigated anti-Gal titers in inflammatory bowel disease (IBD) patients and healthy adult subjects. All studies reporting anti-Gal AB values in a healthy cohort up to now have been cross-sectional,<sup>11</sup> but it is not known whether anti-Gal titers remain constant over a longer time. Therefore, we measured anti-Gal titers of all isotypes and total Ig content in the serum of healthy subjects weekly over 8 weeks. The results provided the possibility to validate former results. Furthermore, we measured anti-Gal AB titers in the plasma of patients suffering from IBD, a disease class that is characterized by an impaired gut barrier. IBD includes two related diseases, namely Crohn's disease (CD) and ulcerative colitis (UC). They produce chronic inflammation in the gastrointestinal tract of unknown origin. Extensive research in the last decades has revealed that the commensal flora, usually tolerated in the gut, is most likely the trigger of the chronic inflammation in IBD. The hypotheses to explain this false

immune response are hyperpermeable epithelium, false antigen presentation, missing T-regulatory cell activity, or missing induction of anti-inflammatory feedbacks.<sup>12,13</sup> Thus we hypothesized that IBD patients displayed an enhanced immune reaction against alpha-Gal, resulting in increased anti-Gal AB levels.

# MATERIALS AND METHODS

## Demographics

*Longitudinal observation of healthy subjects.* We recruited 20 young healthy volunteers for weekly blood samples over 8 weeks to obtain normal values and information about the course of anti-Gal AB isotype titers. Inclusion criteria were an age between 18 and 30 years and good physical health. Exclusion criteria were acute illness or antibiotic intake at the beginning of the study or, chronic illness, immunomodifying medications, vaccinations, or blood transfusions during the study (Table 1). This study was approved by the local ethics committee and conducted in accordance with the Helsinki Declaration of 1975 (as revised in 1983).

*IBD.* As an exemplary disease cohort we chose IBD patients suffering from CD ( $n = 20$ ) or UC ( $n = 20$ ). Details according to the Montreal Classification<sup>14</sup> are displayed in Table 1.

## Enzyme-Linked Immunosorbent Assay

*Alpha-Gal-specific/Antibodies.* The enzyme-linked immunosorbent assay (ELISA) technique was used to measure anti-Gal ABs of all isotypes (IgM, IgG, IgA, IgE, IgD). Plates were coated with Gal $\alpha$ 1-3Gal $\beta$ 1-4GlcNAc Bovine Serum Albumin (Dextra Laboratories, Reading, UK) at 4°C overnight. After rinsing, we added the blocking buffer (phosphate buffered saline -/- + 0.5% bovine serum albumin + 0.05% Tween20). After another rinse step and incubation with the samples, we added detection ABs (goat anti-human IgM, IgG, IgA, IgD polyclonal antibody, goat, all horseradish peroxidase conjugated; Bethyl Laboratories, Montgomery, Tex, USA) and Mouse Alkaline Phosphatase anti-Human IgE Antibody (Becton Dickinson Pharmingen, San Diego, Calif, USA). A color reaction was obtained with peroxidase reagent TMB (3,3', 5,5-tetramethylbenzidine; Sigma-Aldrich, St Louis, Mo, USA) and optical density read at 450 nm using a Victor 3 plate reader (1420 Multilabel Counter, PerkinElmer, Waltham, Mass, USA). For IgE

**Table 1. Demographics**

| Variables                     | Healthy Cohort     |    | UC Patients      | CD Patients     |
|-------------------------------|--------------------|----|------------------|-----------------|
| <i>n</i>                      | 20                 |    | 20               | 20              |
| Sex (F:M)                     | 5:15               |    | 13:7             | 10:10           |
| Age at diagnosis $\pm$ SD (y) | 25.1 $\pm$ 2.9     |    | 24.7 $\pm$ 10.9  | 31.1 $\pm$ 12.0 |
| CRP $\pm$ SD (mg/dL)          |                    |    | 0.85 $\pm$ 3.21  | 0.64 $\pm$ 0.92 |
| Location                      |                    |    |                  |                 |
|                               | UC                 |    | CD               |                 |
|                               | Proctitis          | 2  | Ileal            | 7               |
|                               | Left-sided colitis | 7  | Colonic          | 5               |
|                               | Extensive colitis  | 11 | Ileocolonic      | 8               |
|                               |                    |    | Upper disease    | 2               |
| Behavior                      |                    |    | CD               |                 |
|                               |                    |    | Inflammatory     | 11              |
|                               |                    |    | Strictureing     | 3               |
|                               |                    |    | Penetrating      | 6               |
|                               |                    |    | Perianal disease | 9               |

UC, ulcerative colitis; CD, Crohn's disease; SD, standard deviation; CRP, C-reactive protein.

(pNPP) (4-nitrophenyl phosphate disodium salt hexahydrate, Sigma-Aldrich) was used for a color reaction with optical density read at 405 nm.

As no human anti-Gal AB is commercially available, we were not able to express total anti-Gal Ig content. We used a dilution series of one sample serum on every ELISA plate as an internal standard fitting the curves for interassay comparisons using Origin 8.1 (OriginLab Corporation, Northampton, Mass, USA) and Excel 2003 (Microsoft Corporation, Redmond, Wash, USA).

**Total Ig Isotypes.** ELISA kits were used to measure the total content of Ig isotypes (Human ELISA IgG, IgM, IgA, IgD, IgE Quantitation Set, Bethyl Laboratories). The assay was performed following the instructions as the manufacturers' manual.

Statistical Analysis

**Time Courses of Anti-Gal Titers in Healthy Subjects.** For visualization, we plotted time courses of AB concentrations. We calculated confidence intervals for the slope of a random intercept model to obtain likely ranges of trends. Intraclass correlation coefficients were computed to compare the variances within and among the probands. As a secondary analysis, not correlated different alpha-gal specific AB isotype concentrations with calculation of correlations to their corresponding total concentrations. Pearson correlation coefficients are listed for each week as well as an overall correlation coefficient (corrected for repeated observations).<sup>15</sup> Furthermore, we investigated the linear influence of gender on isotype concentrations.

**AB Concentrations in Healthy and IBD Cohorts.** For comparisons, we computed the mean titers of healthy subjects. Student *t*-test was used to compare groups with results corrected using the Bonferroni-Holm procedure. Pearson correlation was performed to compute correlations, and linear regression, to assess dependencies between parameters. All calculations were performed using R and SPSS 18.0.

RESULTS

Longitudinal Titer Observation in Healthy Adults

For data description, we calculated confidence intervals for the slope of a random intercept model for all measured values of each AB isotype to obtain likely ranges of trends (Table 2). In general, the value 0 for the slopes was overlapped by narrow confidence intervals in the computed models for Alpha-Gal-specific IgG, IgM, IgD, and IgA titers. Thus, these titers remained invariate over the study period within a narrow range (Fig 1).

Only two of the probands displayed Alpha-Gal-specific IgE over the whole period and two showed intermediate positive values. The positive titers varied over a wide range,

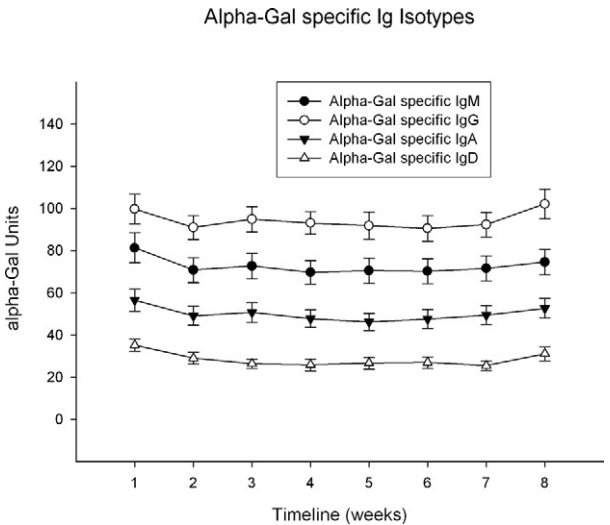


**Fig 1.** Alpha-Gal-specific isotype titers are depicted (expressed in arbitrary alpha-Gal units); mean values  $\pm$  standard error of the mean per time point are plotted. No significant changes can be detected (see Table 2 for confidence interval estimates).

correlating strongly with the total IgE titers of the respective probands (data not shown).

Furthermore, intraclass correlation coefficients were computed to compare variances within and among the probands. Again apart from IgE, the intraclass correlation coefficients were above 0.80, so the variance between the probands overtoped that within the probands. Thus, the titer levels between the subjects varied widely, which held true for every isotype (Table 2).

We estimated the correlation among alpha-Gal-specific Ig titers and to corresponding total Ig titers correcting for repeated observations. Correlations between specific ABs were observed between IgM and the IgA and IgG isotypes (correlation = 0.59 and 0.60, respectively; both  $P < .001$ ). Total and specific Ig titers correlated only in the case of IgM (correlation = 0.28;  $P < .01$ ).

As our data indicated, a substantial difference of alpha-Gal-specific IgG, IgM, and IgA titer levels was assumed between male and female subjects. After a multiplicity correction, there was no significance.

**Table 2. CIs and ICCs of Titer Time Courses in Healthy Adults**

| Variables | CIs          |        |        | ICCs          |       |       |
|-----------|--------------|--------|--------|---------------|-------|-------|
|           | CI Estimates | Lower  | Upper  | ICC Estimates | Lower | Upper |
| IgG       | −0.001       | −0.008 | 0.010  | 0.90          | 0.83  | 0.95  |
| IgM       | −0.006       | −0.013 | 0.002  | 0.94          | 0.90  | 0.97  |
| IgA       | −0.004       | −0.011 | 0.002  | 0.93          | 0.87  | 0.97  |
| IgD       | −0.005       | −0.010 | −0.001 | 0.82          | 0.71  | 0.91  |
| IgE       | −0.049       | −0.082 | −0.016 | 0.56          | 0.39  | 0.75  |

CI estimates plus lower and upper values of the slope of the random intercept model for every isotype are shown. ICCs show a great variance between the probands compared to the variance within each proband. CI, confidence interval; ICC, intraclass correlation coefficients; Ig, immunoglobulin.

# Anti-Gal Antibody Titers in IBD Patients

We measured alpha-Gal-specific IgM, IgG, and IgA AB titers and respective total Ig titers among CD and UC patients. CD patients displayed significantly higher anti-Gal IgA values compared with healthy controls ( $P < .01$ , Fig 2). There were no differences in total Ig concentrations between UC and CD, but both groups showed significantly lower IgM values compared with healthy controls (Table 3). In concordance with results in the healthy cohort, the only correlation between total and specific Ig titer height in the IBD cohort was detected for IgM (correlation = 0.48;  $P < .01$ ). No linear relationship was observed between AB titers and clinical data.

## DISCUSSION

In the present study, we first measured alpha-Gal-specific serum ABs of all isotypes weekly over 8 weeks in 20 young healthy adults. We ascertained that IgG, IgM, IgA, and IgD remained at constant levels in each proband. In contrast, the titers among the probands varied widely, which coincided with the literature.<sup>11</sup> There was a slight decrease in all titers between week 1 and week 2, for which we could not compute a correlation.

Gal-specific ABs of the IgE isotype were negative in most subjects but two had positive values at some times and two were positive over the whole period, albeit varying widely from week to week. These two subjects also showed high total IgE levels. That course correlated strongly with alpha-Gal-specific IgE. Of course, one must consider the limitation of low sample numbers in our observations.

IgM was the only isotype correlating significantly with the specific and total titer course. Moreover, specific IgM correlated with specific IgG and IgA, which may reflect that anti-Gal responses are actually provoked by gut bacteria, as

**Table 3. Anti-Gal and total AB concentrations**

| Variables                      | Control Subjects | UC Patients  | CD Patients   |
|--------------------------------|------------------|--------------|---------------|
| Anti-Gal ABs (alpha-Gal units) |                  |              |               |
| IgG                            | 94.4 ± 26.8      | 79.3 ± 20.8  | 87.6 ± 29.6   |
| IgM                            | 72.6 ± 26.1      | 79.4 ± 23.1  | 78.9 ± 21.1   |
| IgA                            | 51.8 ± 22.7*     | 68.9 ± 37.0  | 80.6 ± 36.6*  |
| Total ABs (mg/mL)              |                  |              |               |
| IgG                            | 11.46 ± 4.55     | 12.37 ± 5.17 | 14.32 ± 10.51 |
| IgM                            | 1.05 ± 0.65*†    | 0.63 ± 0.31† | 0.50 ± 0.45*  |
| IgA                            | 4.35 ± 2.87      | 4.20 ± 2.61  | 3.04 ± 2.62   |

All antibody (AB) concentrations of inflammatory bowel disease patients and controls are quoted. Anti-Gal immunoglobulin (Ig), is significantly increased titers in Crohn's disease (CD) patients compared to healthy control subjects (\* $P < .01$ ). Both CD and ulcerative colitis (UC) patients display significantly decreased levels of total IgM compared to controls († $P < .05$ , \* $P < .01$ , respectively).

it is the case with many other specific ABs. Total IgM serum concentration dynamics could be markedly influenced by this commensal-host interaction through germinal center reactions. Serum AB production of the IgG and IgA isotype would then depend upon stimulation through isotype switching from IgM, which would explain these correlations.

Our data indicated higher titer levels of IgG, IgM, and IgA among women, already reported by Buonomano and colleagues.<sup>11</sup> Our experimental design was inappropriate to calculate significances for such correlations, but we were merely able to indicate a trend. Explanations for the gender difference cannot be drawn from existent data, from our point of view. From these results, it can be assumed that anti-alpha-Gal ABs of IgM, IgG, IgD, and IgA isotypes do not change substantially over longer times, and that significant titer changes actually depict increased antigen exposure with a specific anti-alpha-Gal response.

We hypothesized that the impaired gut barrier in IBD patients results in increased exposure to alpha-Gal bearing bacteria and consequently higher anti-Gal AB titers. We measured anti-Gal-specific IgG, IgM, and IgA and respective total Ig concentrations among patients suffering CD or UC. Alpha-Gal-specific IgA was greatly increased in CD patients compared to our healthy cohort. The anti-Gal IgA titer increase among the UC cohort did not reach significance, a finding that must be confirmed in a larger cohort to identify its potential as a marker protein. Furthermore, we measured total Ig content. Solely anti-Gal-specific and total IgM correlated significantly, confirming results in our healthy cohort. IBD patients displayed significantly lower IgM titers, an observation that was consistent with earlier findings.<sup>16,17</sup>

One may question the importance of these results, as several antigens and specific antibodies have been identified in CD.<sup>18,19</sup> In view of the fact that anti-alpha-Gal ABs are abundant exceeding all other specific ABs by far, their involvement may impact the pathogenesis of disease. Because both diseases have an auto immune component, the

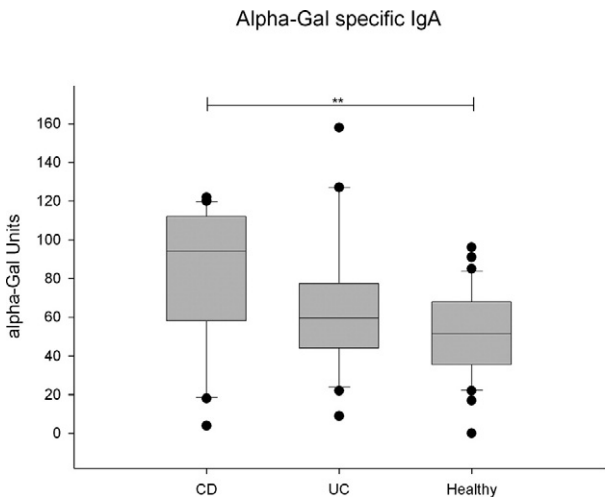


**Fig 2.** Alpha-Gal-specific immunoglobulin (Ig)A of all groups is depicted (expressed in arbitrary alpha-Gal units). Crohn's disease (CD) patients display significantly increased titers compared to healthy subjects (\*\* $P < .01$ ). UC, ulcerative colitis.

immune damage to the end organ may either reflect an attack by ABs or the AB presence may reflect the disease due to bacterial exposure.

## REFERENCES

- Galili U, Rachmilewitz EA, Peleg A, et al: A unique natural human IgG antibody with anti-alpha -galactosyl specificity. *J Exp Med* 160:1519, 1984
- Lai L, Kolber-Simonds D, Park KW, et al: Production of alpha-1,3-galactosyltransferase knockout pigs by nuclear transfer cloning. *Science* 295:1089, 2002
- Mangold A, Szerafin T, Hoetzenecker K, et al: Alpha-Gal specific IgG immune response after implantation of bioprostheses. *Thorac Cardiovasc Surg* 57:191, 2009
- Konakci KZ, Bohle B, Blumer R, et al: Alpha-Gal on bioprostheses: xenograft immune response in cardiac surgery. *Eur J Clin Invest* 35:17, 2005
- Lila N, McGregor CG, Carpentier S, et al: Gal knockout pig pericardium: new source of material for heart valve bioprostheses. *J Heart Lung Transplant* 29:538, 2010
- Mangold A, Ankersmit HJ: Questionable model choice in valve calcification research addressing alpha-Gal. *J Heart Lung Transplant* 29:911, 2010 discussion 2-3.
- Koren E, Neethling FA, Richards S, et al: Binding and specificity of major immunoglobulin classes of preformed human anti-pig heart antibodies. *Transpl Int* 6:351, 1993
- Commins SP, Satinover SM, Hosen J, et al: Delayed anaphylaxis, angioedema, or urticaria after consumption of red meat in patients with IgE antibodies specific for galactose-alpha-1,3-galactose. *J Allergy Clin Immunol* 123:426, 2009
- Mosedale DE, Chauhan A, Schofield PM, et al: A pattern of anti-carbohydrate antibody responses present in patients with advanced atherosclerosis. *J Immunol Methods* 309:182, 2006
- Dahl K, Kirkeby S, d'Apice AF, et al: The bacterial flora of alpha-Gal knockout mice express the alpha-Gal epitope comparable to wild type mice. *Transpl Immunol* 14:9, 2005
- Buonomano R, Tinguely C, Rieben R, et al: Quantitation and characterization of anti-Galalpha1-3Gal antibodies in sera of 200 healthy persons. *Xenotransplantation* 6:173, 1999
- Nagler-Anderson C: Man the barrier! Strategic defences in the intestinal mucosa. *Nat Rev Immunol* 1:59, 2001
- Xavier RJ, Podolsky DK: Unravelling the pathogenesis of inflammatory bowel disease. *Nature* 448:427, 2007
- Silverberg MS, Satsangi J, Ahmad T, et al: Toward an integrated clinical, molecular and serological classification of inflammatory bowel disease: Report of a Working Party of the 2005 Montreal World Congress of Gastroenterology. *Can J Gastroenterol* 19 (suppl A):5, 2005
- Bland JM, Altman DG: Calculating correlation coefficients with repeated observations: Part 1— correlation within subjects. *BMJ* 310:446, 1995
- Di Sabatino A, Carsetti R, Rosado MM, et al: Immunoglobulin M memory B cell decrease in inflammatory bowel disease. *Eur Rev Med Pharmacol Sci* 8:199, 2004
- Soppi E, Granfors K, Leino R: Serum secretory IgA, IgA1 and IgA2 subclasses in inflammatory bowel and chronic liver diseases. *J Clin Lab Immunol* 23:15, 1987
- Lodes MJ, Cong Y, Elson CO, et al: Bacterial flagellin is a dominant antigen in Crohn disease. *J Clin Invest* 113:1296, 2004
- Sandborn WJ: Serologic markers in inflammatory bowel disease: state of the art. *Rev Gastroenterol Disord* 4:167, 2004