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TITLE:  
Proteomic Mapping of the Immune Response to Gluten in Children with Autism

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There is emerging evidence that immune system abnormalities are associated with symptoms in a substantial number of affected individuals. Recent studies point to a role for gastrointestinal (GI) symptoms and defects in GI function in the context of autism. Our newly published data indicate that children with autism exhibit significantly elevated antibody reactivity to gluten, which is associated with GI symptoms. However, our data show that the immune response to gluten in ASD is distinct from celiac disease and involves a different mechanism. The central hypothesis of the proposed study is that the antibody response to gluten in ASD differs significantly from celiac disease, targeting a unique set of proteins and epitopes that can be utilized to identify novel biomarkers of the condition and gain novel insights regarding mechanism. The activities in the first year of the grant period have been focused on the first proposed Aim, i.e., characterizing the specific target molecules of the anti-gluten antibody response in ASD. As proposed, we have completed the construction of arrays and are now in the process of the microarray analysis of the antibody data. We have also completed the 1D immunoblotting analysis of the antibody responses to both gluten and nongluten proteins of wheat in patients and controls. Details of the results are presented in this report.
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1. INTRODUCTION:

Autism spectrum disorders (ASD) are a heterogeneous group of neurodevelopmental diseases characterized by deficits in communication skills and social interaction, as well as the presence of repetitive and stereotyped patterns of behavior. The reported prevalence of ASD has increased sharply over the last few decades to greater than 1%, while the pathogenic mechanisms of the disease remain largely unknown and effective treatment options are limited. In addition, a clear barrier to the better understanding of ASD has been the heterogeneity within this spectrum and the lack of biomarkers to characterize disease phenotypes and to understand treatment outcome. There is emerging evidence that immune system abnormalities are associated with symptoms in a substantial number of affected individuals. Recent studies point to a role for gastrointestinal (GI) symptoms and defects in GI function in the context of autism. Dietary gluten, a group of over 70 different proteins in wheat and related cereals, has been suspected of having a role in some patients with ASD. Diets that exclude gluten are increasingly popular in the autism community, although their effectiveness has not been proven in controlled studies. Our newly published data indicate that children with autism exhibit significantly elevated antibody reactivity to gluten, which is associated with GI symptoms. The data also show that the immune response to gluten in ASD is distinct from celiac disease (an autoimmune disorder triggered by gluten) and involves a different mechanism. The central hypothesis of the proposed study is that the immune response to gluten in ASD is fundamentally different from celiac disease, targeting a unique set of proteins and epitopes, which can be utilized to identify novel biomarkers and gain novel insights about mechanism. The specific aims of this proposal, which represent a systematic approach to examine this hypothesis, are: 1) to fully characterize the specific target molecules of the anti-gluten antibody response in ASD, using a gluten protein/peptide microarray system, and 2) to map the specific target sequences of the identified gluten proteins associated with the antibody response in ASD. If the aims of the proposed project are achieved, the results would 1) offer biomarkers that may be useful in identifying subsets of ASD patients or individuals at risk of developing ASD, 2) support the examination of specific treatment strategies, including gluten exclusion diet, targeted at the identified subset of patients, and 3) yield experimental support for closer examination of the role of the identified proteins or the immune response to them in the pathogenesis of the disorder.

2. KEY WORDS:

Autism, immune response, gastrointestinal symptoms, antigen, gluten, IgG, antibody, microbiota

3. ACCOMPLISHMENTS:

3.1. What were the major goals of the project?

The major goals of this project, as stated in the approved SOW were as follows:
Aim 1. To characterize the molecular specificity of the antibody response to gluten in autism, using a novel gluten proteomic microarray system (Year 1, months 1-12; Year 2, months 1-9).
Task 1.1. Acquisition of patient and control serum samples (Year 1, month 1).
Task 1.2. Measurement of levels of antibody to gluten, deamidated gliadin, and TG2 (Year 1, month 1).
Task 1.3. Construction of the gluten proteomic microarray containing the full set of immunogenic gluten proteins (months 3-8).
  Task 1.3.1. Extraction of gluten proteins (Year 1, month 2).
  Task 1.3.2. Digestion of extracted gluten proteins (Year 1, month 2).
  Task 1.3.3. Reversed-phase HPLC separation of gluten proteins and gluten digests (Year 1, months
Task 1.3.4. Fractionation and processing of separated gluten proteins and peptides (Year 1, month 5).

Task 1.3.5. Printing of fractionated gluten proteins/protein fragments and array optimization (Year 1, months 6-7).

Task 1.4. Gluten microarray antibody analysis (Year 1, months 8-12; Year 2, month 1).

Task 1.5. Determination of identity of target proteins by LC-MS/MS-assisted peptide mass mapping (Year 2, months 2-4).

Task 1.6. Mapping of antibody target profile by two-dimensional immunoblotting and proteome mapping (Year 2, months 5-10).

Task 1.6.1. Two-dimensional separation of gluten proteins (Year 2, months 5-6).

Task 1.6.2. Immunoblotting (Year 2, months 7-10).

Task 1.6.3. Identification of reactive gluten proteins (Year 2, months 8-12).

Task 1.7. Correlation of antibody levels with subject cohort and ASD symptoms (Year 2, month 12).

Aim 2. To map the epitopes of target gluten proteins associated with the antibody response in autism (Year 3).

Task 2.1. Peptide microarrays (Year 3, months 1-2).

Task 2.2. Epitope mapping (Year 3, months 3-6).

Task 2.3. Development of ELISA for confirmation of target epitopes (Year 3, months 7-9).

Task 2.4. Correlation of antibody levels with ASD symptoms (Year 3, month 10).

Task 2.5. Additional data analysis and preparation of manuscript(s) (Year 3, months 10-12).

3.2. What was accomplished under these goals?

We have completed all of the tasks proposed in the SOW for this period, as follows:

1. Acquired and aliquoted patient and control serum samples.
2. Completed the extraction of gluten proteins from wheat.
3. Completed the digestion of extracted gluten proteins.
4. Completed the reversed-phase HPLC separation of gluten proteins and gluten digests. The pattern of chromatographic separation is shown in Fig. 1.
5. Completed the fractionation and processing of separated gluten proteins and peptides.
6. Completed the printing of fractionated gluten proteins/protein fragments.
7. We are now in process of the gluten microarray antibody analysis. This work is underway.
8. We are in the process of one-dimensional immunoblotting analysis of the antibody response to gluten and nongluten proteins of wheat. Fig. 2 shows the analysis of target reactivity of antibody response in children with autism (n=11), unaffected siblings (n=11), and healthy children (n=11), found to have high antibody response to gluten by ELISA. The immunoblotting analysis confirms that the children with autism have the strongest reactivity to gluten proteins, primarily in the 30-50 kDa range. The gluten proteins in this range are composed mostly of α-gliadin and γ-gliadin proteins, as well as some low molecular
weight glutenin proteins. The planned two-dimensional blotting, as well as the ongoing microarray analysis will identify the exact protein targets.

3.3. What opportunities for training and professional development has the project provided?

Nothing to report.
3.4. How were the results disseminated to communities of interest?
   Nothing to report.

3.5. What do you plan to do during the next reporting period to accomplish the goals?
   The following tasks will be completed during the second of year of the proposed study, as outlined in the SOW.
   Task 1.4. Gluten microarray antibody analysis (Year 1, months 8-12; Year 2, month 1).
   Task 1.5. Determination of identity of target proteins by LC-MS/MS-assisted peptide mass mapping (Year 2, months 2-4).
   Task 1.6. Mapping of antibody target profile by two-dimensional immunoblotting and proteome mapping (Year 2, months 5-10).
      Task 1.6.1. Two-dimensional separation of gluten proteins (Year 2, months 5-6).
      Task 1.6.2. Immunoblotting (Year 2, months 7-10).
      Task 1.6.3. Identification of reactive gluten proteins (Year 2, months 8-12).
      Task 1.7. Correlation of antibody levels with ASD symptoms (Year 2, month 12).

4. IMPACT:

4.1. What was the impact on the development of the principal discipline(s) of the project?
   Nothing to report.

4.2. What was the impact on other disciplines?
   Nothing to report.

4.3. What was the impact on technology transfer?
   Nothing to report.

4.4. What was the impact on society beyond science and technology?
   Nothing to report.

5. CHANGES/PROBLEMS:

5.1. Changes in approach and reasons for change.
   Nothing to report.

5.2. Actual or anticipated problems or delays and actions or plans to resolve them.
   Nothing to report.

5.3. Changes that had a significant impact on expenditures.
   Nothing to report.

5.4. Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents.
   Nothing to report.

5.5. Significant changes in use or care of human subjects.
   Nothing to report.
5.6. Significant changes in use or care of vertebrate animals.
   Nothing to report.

5.7. Significant changes in use of biohazards and/or select agents.
   Nothing to report.

6. PRODUCTS:

5.1. Publications, conference papers, and presentations.
   Nothing to report.

5.2. Website(s) or other Internet site(s).
   Nothing to report.

5.3. Technologies or techniques.
   Nothing to report.

5.4. Inventions, patent applications, and/or licenses.
   Nothing to report.

5.5. Other Products.
   Nothing to report.

6. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

6.1. What individuals have worked on the project?
   1) Name: Armin Alaedini, PhD
      Project role: PI
      Nearest person months worked: 4
      Contribution to project: Dr. Alaedini has overseen the completion of the proposed tasks, as was described in the proposal, including acquisition of samples, interaction with project collaborators, analysis of data, and hands on assistance of the research scientists in the laboratory.

   2) Name: Mary Ajamian, MS
      Project role: Research assistant
      Nearest person months worked: 4
      Contribution to project: Ms. Ajamian has been involved with all the laboratory aspects of the project, including the completion of the ELISA and immunoblotting analyses.

6.2. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?
   Yes. The changes to the PI’s active support are as follows. These changes do not affect the PI’s effort on the current grant.

   A. Previously active grants that have closed:
“Northeast Biodefense Center: Molecular Biomarkers for Identification of Disease Stage in Lyme Borreliosis”

“Immunologic mechanisms in post-Lyme disease syndrome”

B. New grant support:
20002598 Verdu (PI); Alaedini (Subaward PI) 06/01/2015-05/31/2016
Title: The effect of gluten-free diet on gastrointestinal motility in patients with gluten-sensitive IBS
Subaward title: Molecular specificity of immune response to wheat in IBS
Source: Canadian Institutes of Health Research/McMaster University

Nxt2b Grant Award Alaedini (PI) 11/1/2014-10/30/2017
Title: Immunologic mechanisms in cerebral palsy
Source: Nxt2b AB

6.3. What other organizations were involved as partners?
   Location of Organization: New York, NY.
   Partner’s contribution to the project: Provided serum samples.
   Financial support: None.
   In-kind support: None.
   Facilities: Project staff do not use the facilities of WCARP.
   Collaboration: WCARP staff and director are involved in the discussion of the research results.
   Personnel exchanges: None.

2. Organization Name: Autism Speaks
   Location of Organization: New York, NY.
   Partner's contribution to the project: Provided serum samples.
   Financial support: None.
   In-kind support: None.
   Facilities: None.
   Collaboration: Other than providing serum samples for the project, there is no other interaction with Autism Speaks.
   Personnel exchanges: None.

3. Organization Name: Celiac Disease Center at Columbia University
   Location of Organization: New York, NY.
   Partner's contribution to the project: Provided serum samples.
   Financial support: None.
   In-kind support: None.
   Facilities: None.
   Collaboration: Celiac Disease Center’s director is involved in the review of the data and the writing of manuscripts.
   Personnel exchanges: None.