Serologic Reactivity to the Emerging Pathogen *Granulibacter bethesdensis*

David E. Greenberg,^{1,a} Adam R. Shoffner,¹ Kimberly R. Marshall-Batty,^{1,a} Kriti Arora,¹ Ming Zhao,⁴ Raynaldo Martin,⁴ Li Ding,¹ Carl H. Hammer,⁴ Pamela A. Shaw,³ Douglas B. Kuhns,⁵ Harry L. Malech,² John I. Gallin,² Kol A. Zarembek,² and Steven M. Holland¹

¹Laboratory of Clinical Infectious Diseases; ²Laboratory of Host Defenses; ³Biostatistics Research Branch, National Institute of Allergy and Infectious Diseases (NIAID), Bethesda; ⁴Research Technologies Branch, NIAID, Rockville; and ⁵Clinical Services Program, SAIC-Frederick, Frederick, Maryland

Background. *Granulibacter bethesdensis* is a recently described member of the Acetobacteraceae family that has been isolated from patients with chronic granulomatous disease (CGD). Its pathogenesis, environmental reservoir(s), and incidence of infection among CGD patients and the general population are unknown.

Methods. Detected antigens were identified by mass spectroscopy after 2-dimensional electrophoresis and immunoaffinity chromatography. The prevalence of *Granulibacter* immunoreactivity was assessed through immunoblotting and enzyme-linked immunosorbent assay (ELISA).

Results. Methanol dehydrogenase (MDH) and formaldehyde-activating enzyme were recognized during analysis of sera from infected patients. Unique patterns of immunoreactive bands were identified in *Granulibacter* extracts, compared with extracts of other Acetobacteraceae species. By use of criteria based on these specific bands, specimens from 79 of 175 CGD patients (45.1%) and 23 of 93 healthy donors (24.7%) reacted to all 11 bands. An ELISA that used native MDH to capture and detect immunoglobulin G was developed and revealed high-titer MDH seroreactivity in culture-confirmed cases and 5 additional CGD patients. Testing of samples collected prior to culture-confirmed infection demonstrated instances of recent seroconversion, as well as sustained seropositivity. Infection of CGD mice with *G. bethesdensis* confirmed acquisition of high-titer antibody-recognizing MDH.

Conclusions. These serologic tests suggest that *Granulibacter* immunoreactivity is more common among CGD patients and, perhaps, among healthy donors than was previously suspected. This finding raises the possibility that clinical presentations of *Granulibacter* infection may be underappreciated.

Chronic granulomatous disease (CGD) is an inherited immunodeficiency caused by defects in the phagocyte NADPH oxidase that lead to impaired production of superoxide and its metabolites [1]. CGD patients get recurrent infections that are typically caused by *Staphylococcus aureus*, *Serratia marcescens*, *Burkholderia cepacia* complex, *Nocardia* species, and *Aspergillus* species [2–6]. *Granulibacter bethesdensis* was originally

isolated from a CGD patient with fever and lymphadenopathy and has since been isolated from at least 6 CGD patients [7–10]. Of interest, only 1 patient is known to have died from this organism [9]. In CGD mice, *G. bethesdensis* causes long-term infection with pathologic changes, while in wild-type mice, this organism appears nonpathogenic but could be cultured from spleen 76 days after infection.

Granulibacter organisms belong in the Acetobacteraceae, a diverse family that includes the acetic acid bacteria. Acetobacteraceae species are found worldwide, particularly in tropical regions [11–13], and some are increasingly associated with infection of immunocompromised patients [14–17]. In CGD, the causative pathogen of an infection, including lymphadenitis, is frequently not found despite response to empirical therapy. CGD patients are typically infected by opportunistic pathogens that exist in the

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^aPresent affiliation: University of Texas Southwestern Medical Center, Dallas, Texas.

Correspondence: David Greenberg, MD, University of Texas Southwestern Medical Center, 5323 Harry Hines Blvd, Dallas, TX 75390-9113 (david.greenberg@utsouthwestern.edu).

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environment. We hypothesized that exposure to *Granulibacter* organisms was likely broader than the culture recovery rate reflected. To test this hypothesis, we developed serologic assays for *Granulibacter* organisms to determine the seroprevalence of exposure and to better characterize the antibody responses to this pathogen.

MATERIALS AND METHODS

Protein Extract Preparation

G. bethesdensis National Institutes of Health (NIH) strains NIH1.1 (strain CGDNIH1^T; ATCC BAA-1260), NIH2.1, NIH2.2, NIH3.1, and NIH4.1 [10] were cultured in yeast peptone glucose (YPG) medium (5 g of yeast extract, 3 g of peptone, and 10 g of glucose per liter of water). Single colonies were inoculated into 5 mL YPG, shaken overnight (at 180 rpm and 37°C), and subcultured into 150 mL of YPG for 48 hours. Bacteria were washed in 150 mM NaCl, followed by centrifugation (for 10 minutes at 4°C and 5000 × g) 3 times. Pellets were stored at −80° C and were then weighed, resuspended in 3 mL 150 mM NaCl per gram, and sonicated 6 times (30 seconds/pulse) on ice at power level 2, using a Sonifier 150 (Branson). *Acetobacter estunensis* ATCC 23753, *Gluconobacter oxydans* ATCC 621H, *Asaia bogorensis* ATCC BAA-21, *Acidomonas methanolica* ATCC 43581, and *Acetobacter orleanensis*

ATCC 12876 were grown at 30°C and processed as above. Sonicated extracts were centrifuged at 21 000 × g (for 30 minutes at room temperature). Protein concentration was determined using the Quick Start Bradford Dye Reagent (Bio-Rad).

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis

For immunoblot screening, solubilized bacterial extracts were pooled by mixing equal amounts (based on protein content) from NIH1.1, NIH2.1, NIH3.1, and NIH4.1. After heating (for 5 minutes at 95°C in NuPAGE LDS buffer with 5% β-mercaptoethanol), 2 μg of this pool per lane was resolved on 4%–12% SDS-PAGE Bis-Tris gels in MOPS buffer (Invitrogen), transferred to nitrocellulose, and blocked overnight at 4°C in Tris-buffered saline (pH 7.4) with 0.05% Tween-20 (TBST) containing 5% (w/v) bovine serum albumin (Millipore). Sera or plasma specimens were diluted at 1:250 in blocking buffer, and membranes were incubated at room temperature for 1 hour. After 3 TBST washes, the membrane was incubated with horseradish peroxidase-conjugated goat anti-human immunoglobulin G (IgG; dilution, 1:10 000; Amersham). Blots were developed using ECL Plus (Amersham) and exposed for 10, 30, and 60 seconds. On the basis of initial experiments using sera from the culture-confirmed patients, an immunoblot was considered positive if 11 bands (Figure 1) were detectable at a serum dilution of 1:250 within 60 seconds of exposure.

Figure 1. Immunoblots performed using serum from the 4 infected patients with chronic granulomatous disease against pooled *Granulibacter* extracts. The stars on the left of the blots denote the 11 bands subsequently used to determine seropositivity. *A*, Sera from patients 1 and 3 and negative control sera were used at a 1:250 dilution. Serum from patient 4 was used at 1:1000 dilution. Serum from patient 2 was used at a 1:5000 dilution. *B*, Titration of serum from patient 2.

Human Samples

Plasma specimens from healthy donors (age, ≥ 18 years) and sera and plasma specimens from CGD patients were obtained and stored in $N_2(l)$, using existing institutional review board–approved protocols. This test set included 175 samples from CGD patients without clinically confirmed *Granulibacter* infection. Five patients with culture-confirmed *Granulibacter* infection served as positive controls.

Two-Dimensional Gel Electrophoresis

After isoelectric focusing to equilibrium (at 30 000 V for 1 h) by use of IPG ZOOM strips (pH 3–10; Invitrogen), separation by molecular weight was performed in NuPAGE 4%–12% Bis-Tris ZOOM gels (Invitrogen), and protein bands were stained with Coomassie Brilliant Blue or transferred to nitrocellulose. Membranes were blocked in skim milk (10% w/v) in TBST, incubated with serum (dilution, 1:1000), washed, and incubated with HRP-linked sheep anti-human IgG (dilution, 1:2000; GE Healthcare). The membrane was developed (SuperSignal West Pico chemiluminescent system, Pierce) and reused for incubation with each patient's serum separately and comparison with the paired Coomassie Brilliant Blue–stained gel. The membrane was incubated with 2% SDS and 20 mM dithiothreitol (at 65°C for 1 hour) with shaking to remove bound antibodies, rinsed with water, reblocked, and incubated with sera from other patients, as described above. If diminishing signal on immunoblot developed with reuse of the blot, new sets of gels were run.

Purification of IgG and *Granulibacter* Antigens

Human IgG was prepared by affinity chromatography, using a 1-mL HiTrap protein G HP column (GE Healthcare). IgG-rich fractions were eluted with low-pH glycine buffer and collected into 1 M Tris (pH 8.0). Eluted material was desalted into phosphate-buffered saline (PBS), using a Sephadex G-25 column (PD-10, GE), and filtered (0.2 μ m). The protein concentration was determined using a Nanodrop spectrophotometer (Thermo Scientific), or protein was concentrated with an Amicon Ultra-4 10K MWCO ultrafiltration device (Millipore). The buffer of the antibody solution was changed to coupling solution (0.2 M NaHCO_3 and 0.5 M NaCl; pH 8.3), diluted, and centrifuged 6 times with the same ultrafiltration device. Concentrated antibodies (approximately 0.8 mL in the coupling buffer) were immobilized onto a 1-mL HiTrap NHS-activated HP column (GE) and blocked in accordance with the manufacturer's recommendations. Approximately 90% of applied antibodies were immobilized onto the column.

For each column, *Granulibacter* extracts were prepared as described above except that, prior to sonication, 5 mL of lysis buffer (10 mg CHAPS, 1 $\times$ PBS, and 1 Roche protease inhibitor cocktail tablet) were added to each pellet. The supernatant containing the soluble protein fraction (approximately 2.5 mg)

was diluted to approximately 2 mL and applied onto the column. Unbound fractions were washed out with PBS–0.2% CHAPS, and bound material was eluted using 0.5 M glycine (pH 2.7). Eluates were neutralized, concentrated, and applied onto the SDS-PAGE gel (4%–12%, MOPS buffer, Invitrogen). Gels were stained with Coomassie Brilliant Blue, and bands were excised and processed for mass spectroscopy (MS). For these immunoaffinity experiments, protein extracts of each isolate were run through the column individually.

MS and Protein Identification

One- or two-dimensionally separated proteins were reduced, alkylated, digested with trypsin, and identified by standard MS protocols. For some samples, tryptic digests were separated using a Zorbax C18SBW reverse-phase column (0.15 mm ID $\times$ 100 mm). The mobile phase consisted of a gradient prepared from solvent A (0.2% formic acid) and solvent B (99.8% acetonitrile and 0.2% formic acid) at room temperature (flow rate, 1 μ L/minute). For these fractionated digests, capillary liquid chromatography–tandem MS was performed with a CapLC and quadrupole time-of-flight MS QToF-2 (Waters Micromass). Computer-controlled, data-dependent automated switching to MS/MS provided peptide-sequence information. MassLynx and Global Server software were used for data acquisition and processing.

The majority of tryptic digests were analyzed by coupling the Nanomate (Advion BioSciences), an automated chip-based nano-electrospray interface source, to the QStarXL MS/MS system (Applied Biosystems/Sciex), a quadrupole time-of-flight MS. Data processing and data bank searching were performed with either Mascot software (Matrix Science) or AnalystQS software (Applied Biosystems/Sciex), using the National Center for Biotechnology Information nonredundant protein database.

Purification of Methanol Dehydrogenase (MDH)

We purified MDH from *Granulibacter* organisms essentially as described elsewhere [18]. *Granulibacter* (in the mid-log phase of growth) was harvested (at 4500 $\times$ g for 25 minutes), and pellets were resuspended in water (1 g/4 mL water) and sonicated (20 times for 30 seconds each). Supernatant was diluted at 1:4 in 100 mM morpholino-ethane sulfonic acid (MES; pH 5.50), filtered (using a 0.7- μ m cellulose acetate filter), and applied to a SP-Sepharose HP column (GE Biosciences) pre-equilibrated with 25 mM MES (pH 5.5). Bound material was eluted with a gradient (0–250 mM NaCl in 25 mM MES [pH 5.5]) over 40 minutes (flow rate, 1 mL/minute; bed volume, 5 mL). Fractions analyzed by SDS-PAGE indicated MDH eluted at approximately 50 mM NaCl. Gel filtration was performed on a Superdex 200 10/300 GL column (GE Life Sciences) in 25 mM MES (pH 5.5) with 100 mM NaCl (flow rate, 1 mL/minute). Fractions containing MDH

(subunits 1 and 2; approximately 69 kDa and approximately 11 kDa, respectively, as determined by SDS-PAGE) were pooled and stored at -80°C . MS confirmed the purified proteins as MDH subunits 1 and 2. Silver stain of SDS-PAGE gels showed a purity of approximately 95%.

Enzyme-Linked Immunosorbent Assay (ELISA)

An indirect ELISA was developed using Costar 96-well ELISA plates (Corning) coated with *Granulibacter* MDH diluted in PBS (100 ng/well). After overnight incubation at 4°C , plates were washed (using PBS with 0.05% Tween-20) and then blocked with 5% fetal bovine serum (Thermo Scientific HyClone), PBS, and sodium azide (2 mg/mL) for 1 hour. Donor samples were serially diluted in blocking solution and tested at dilutions ranging from 1:256 to 1:262,144. Sera were incubated in both MDH-coated and uncoated wells (for background subtraction) for 2 hours and were washed with PBST. Sheep anti-human IgG-HRP secondary antibody (GE Healthcare) diluted at 1:1000 in blocking solution was added to each well (100 μL /well), specimens were incubated for 1 hour and washed, and 100 μL of TMB Substrate Reagent (BD Biosciences) was used to detect *Granulibacter* organisms. After 4 minutes, 100 μL of 2 N sulfuric acid was added to stop the reaction. Absorbance at a wavelength of 450 nm (A_{450}) was read on a Beckman Coulter DTX880 Multimode Detector. For titer determination, nonlinear regression analysis was performed using GraphPad Prism (version 5.0). By use of the best-fit curve generated by this analysis, the titer of the serum was determined to be the dose required to achieve an A_{450} of 0.5.

Mouse Experiments

Six male $\text{p47}^{\text{phox-/-}}$ mice approximately 20 weeks old were injected intraperitoneally with 10^8 colony-forming units of strains NIH1.1, NIH2.2, or NIH4.1 (2 mice/strain). Serum was obtained 1 week before infection and at 2, 5, and 29 weeks after infection. ELISA was performed as described above for human samples, except that the first dilution tested was 1:1024 and the sheep anti-mouse IgG secondary was used at a dilution of 1:1000.

Statistical Analyses

For immunoblot analysis, the proportion of individuals having each specific band and having a total of 0–11 bands present was calculated separately for the CGD and healthy groups. The Fisher exact test was used to test for differences in proportions between the 2 groups. The Wilcoxon rank-sum test was used to test whether one group tended to have more bands than the other. For all tests, a 2-sided P value of $\leq .05$ was considered statistically significant. For interpretive purposes, the collection of hypothesis tests for the presence or

absence 11 bands were also done using the Bonferroni correction for multiple comparisons.

RESULTS

Given the recognized genetic differences between the *Granulibacter* isolates to date [10] and the small number of isolates that had been found, we pooled soluble *Granulibacter* extracts from 4 NIH isolates to better represent this diversity for immunoblot screening purposes. Although sera from our patients known to be culture positive [7, 8, 13] showed a variety of bands when tested against these pooled extracts, we consistently detected 11 distinct bands with apparent molecular weights of approximately 150, 140, 100, 80, 75, 70, 62, 51, 45, 38, and 28 kDa. Therefore, we chose recognition of these bands as criteria for seropositivity (Figure 1A and 1B). Although all 11 bands were recognized for samples from each of the 4 patients with culture-proven infection, the relative intensities were highly variable. A less diluted sample (dilution, 1:250) from patients 1 and 3 was required to recognize all 11 bands, while samples from patients 2 and 4 had all 11 bands visible at greater dilutions. The sample from patient 4 had all 11 bands recognized at a dilution of 1:1000 (Figure 1A), and the sample from patient 2 had all 11 bands recognized at a dilution of 1:20 000 (Figure 1B). Therefore, because samples from all 4 patients with culture-proven infection had all 11 bands detected at titers of 1:250 and above, we chose 1:250 as the dilution at which we could reliably detect prior exposure to *Granulibacter* organisms.

To determine whether bands detected by immunoblotting were specific to *Granulibacter* organisms or shared with other Acetobacteraceae species, extracts from the closely related organisms *G. oxydans*, *A. bogorensis*, *A. methanolica*, *A. estunensis*, and *A. orleanensis* were run on SDS-PAGE and probed with sera from patients with culture-proven *Granulibacter* infection (Figure 2). None of these Acetobacteraceae species showed banding patterns that reproduced the patterns seen with *G. bethesdensis*. However, some individual bands had a molecular weight similar to that of some of the bands reactive in *Granulibacter* specimens (Figure 2). Recently, another acetic acid bacterium, *Acidomonas methanolica*, was isolated from the lymph node of a single CGD patient [30]. When serum from this patient was incubated with *Acidomonas* extracts or *Granulibacter* extracts, the observed banding pattern was distinct from that seen in sera from the *Granulibacter*-infected patients. Further studies of this patient will be required to determine the specificity of these assays for these 2 organisms.

To determine the rate of seroreactivity in the NIH CGD cohort and the general population, we performed immunoblotting on specimens from 175 CGD patients who never had culture-proven *Granulibacter* infection and from

Figure 2. Immunoblots comparing *Granulibacter* organisms to other *Acetobacteraceae* species. Serum from patient 1 immunoblotted against pooled whole bacterial extracts of *Granulibacter bethesdensis* isolates (National Institutes of Health [NIH] strains NIH1.1, NIH 2.1, NIH3.1, and NIH4.1), along with similar extracts from 5 closely related *Acetobacteraceae* species. Each lane contains 2 µg total protein. Patient serum was used at a 1:250 dilution.

93 anonymous healthy donors. Specimens from 45% of CGD patients (79 of 175) had all 11 bands recognized on the pooled *Granulibacter* immunoblot, and specimens from 25% of healthy donors (23 of 93) also had 11 bands recognized ($P = .001$, by the Fisher exact test). Among CGD patients, specimens from 87% (152 of 175) had ≥ 8 bands recognized, compared with specimens from 76% of healthy donors (71 of 93) ($P = .039$, by the Fisher exact test). Specimens from CGD patients had a median of 10 bands recognized, compared with 9 bands for healthy donors ($P = .0007$, by the Mann-Whitney U test). Age did not alter the rate of recognition of all 11 bands among CGD patients: specimens from 49 of 107 patients aged >18 years had all 11 bands recognized, as did specimens from 30 of 68 aged <18 years ($P > .05$, by the Fisher exact test). Sera from 4 CGD patients had ≤ 2 bands recognized, while serum from 1 healthy donor had ≤ 2 bands recognized. Because our healthy donors were adults, no age-associated prevalence could be determined. Therefore, almost half of CGD patients and a quarter of healthy subjects had specimens that recognized all *Granulibacter* bands on immunoblotting.

The heavy chain of MDH had been recognized by serum from the first patient with *Granulibacter* infection [7]. However, the identities of the remaining 10 bands were unknown. We used 2-dimensional PAGE, immunoblotting, and MS to identify immunodominant antigens recognized by patients with culture-

confirmed *Granulibacter* infection (Supplementary Figure 1). To confirm the proteins identified by 2-dimensional PAGE, we prepared immunoaffinity columns, using purified patient IgG. After extensive washing, bound antigens were eluted and identified by MS (Supplementary Figure 1). These 2 techniques identified a total of 16 antigens, several of which were detected by both methods (Supplementary Table 1). Among these antigens were the following *Granulibacter* homologues of 5 proteins that have been reported in serologic studies of other bacterial infections: LSU ribosomal protein L12P, elongation factor TU, the chaperonins GroEL and GroES, and DnaK.

Of importance, 4 antigens were related to the ability of *Granulibacter* organisms to metabolize methanol [13]. Antibodies from 4 culture-positive patients recognized MDH1. Three patients' antibodies recognized the other component of this protein complex, MDH2, while serum from patient 4 did not. Along with MDH1 and MDH2, sera from 3 of the patients recognized formaldehyde-activating enzyme, another protein involved in methanol metabolism, and IgG from patient 3 detected the moxJ protein precursor.

Although we detected widespread reactivity to *Granulibacter* organisms in different patients by immunoblotting, immunoaffinity chromatography, and 2-dimensional PAGE/mass spectroscopy, these techniques are not amenable to high-throughput screening. Therefore, we developed an ELISA against MDH, one of the few antigens that appeared to be specific to *Granulibacter* organisms. Native MDH was purified and used in an indirect ELISA to measure MDH-reactive IgG. Sera from 5 CGD patients culture-positive for *Granulibacter* organisms were titrated for MDH immunoreactivity by ELISA (Figure 3A). All 5 patients showed clear IgG responses to MDH at titers $\geq 1:7000$ (range, 1:7293 to 1:67,078). At dilutions of 1:256 and 1:1024, the differences between the culture-positive CGD patients and the healthy subjects were largest. We chose the 1:1024 dilution to screen available sera from CGD patients and healthy donors for MDH immunoreactivity. At the 1:1024 dilution, sera from patients with culture-proven infection had a mean absorbance ($\pm$ SD) of 1.12 ± 0.138 . We defined ELISA-based seropositivity as an absorbance within 3 SDs of the culture-confirmed sample mean (absorbance, ≥ 0.5). By using this criterion, 5 additional CGD patients (2.6%) without known histories of *Granulibacter* infection were identified, and no healthy donors displayed evidence of MDH reactivity (Figure 3B). When fully titrated, these reactive sera from CGD patients ranged from 1:3246 to 1:7640, similar to the titers of sera from patients with culture-proven *Granulibacter* infection.

To determine how soon after acute infection the antibody response to MDH developed, we infected CGD mice with different strains of *G. bethesdensis* (Figure 4). Sera obtained 1 week before infection were negative at a dilution of 1:256, and, regardless of the infecting strain, MDH-reactive IgG was

Figure 3. Human seropositivity determined by enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G against *Granulibacter bethesdensis* methanol dehydrogenase (MDH). *A*, Sera from 5 culture-confirmed cases and 7 healthy donors were titrated. For patients, a 4-parameter nonlinear regression analysis (plotted as solid lines) was performed to model the titer curves based on 73–129 data points per donor (symbols at mean $\pm$ SD). For the healthy donors, the mean and range are plotted. *B*, ELISA screening of human sera for antibodies against MDH at a dilution factor of 1024 (greatest ratio of patients with culture-confirmed infection to healthy donors). Individual absorbances are shown for 79 healthy donors (+), 153 patients with chronic granulomatous disease (o), and 5 patients with culture-confirmed infection ($\bullet$). For the cases, the mean $\pm$ SD is shown, and the black dotted line indicates the cutoff used for determining seropositivity (ie, 3 SDs below the culture-confirmed sample mean).

detected in all mice by 5 weeks after infection. Antibody levels remained elevated for >200 days, although at lower levels than at 5 weeks. Therefore, in CGD mice, MDH detection by ELISA appeared to be a good indicator of recent infection with *G. bethesdensis*.

Seroconversion in human patients was documented using the MDH ELISA to titer archived sera from patients with culture-confirmed infection. Figure 5A shows that MDH ELISA immunoreactivity of serum from patient 5 coincided with the onset of a discreet illness that culminated in the isolation and treatment of *G. bethesdensis*. This patient was seronegative for 18 years before his illness-associated seroconversion. In contrast, patient 3 acquired seroreactive IgG to MDH years before the isolation of *Granulibacter* organisms.

DISCUSSION

In the absence of direct culture, seroprevalence provides important information about the incidence of *Granulibacter* infection in CGD patients and, possibly, the healthy population. Acetobacteraceae species have a global distribution, and some are common in the human environment and diet. Indeed,

Figure 4. Chronic granulomatous disease mouse seropositivity determined by enzyme-linked immunosorbent assay (ELISA) for immunoglobulin G against *Granulibacter bethesdensis* methanol dehydrogenase. p47^{phox} mice were infected with *G. bethesdensis* strains NIH1.1, NIH2.2, NIH3.1, and NIH4.1 (2 mice per strain), as described in Methods, and serum was collected at the indicated times and analyzed by ELISA. High-titer antibody was present by 5 weeks after infection and remained positive >200 days later.

Figure 5. Methanol dehydrogenase antibodies in culture-confirmed *Granulibacter bethesdensis* infections over time. A, Enzyme-linked immunosorbent assay (ELISA) data for patient 5 over a 20-year period prior to diagnosis of *G. bethesdensis* infection in May 2009. B, ELISA data for patient 3 over an approximately 10-year period prior to diagnosis in January 2006. The onset of the patient's clinical illness ascribed to *Granulibacter* infection is shown.

molecular evidence documented the presence of Acetobacteraceae species on the forearm of one individual [19]. Increasingly, infections from other Acetobacteraceae species have been reported in some immunocompromised patients [14–17, 20]. Six culture-positive cases of *G. bethesdensis* infection in CGD, a disease with a molecularly and cellularly defined immune defect, provides a unique opportunity to dissect previously unrecognized host and pathogen determinants of disease.

Because methylotrophic organisms are rare causes of human disease, it is remarkable that we identified several immunodominant antigens involved in the ability of *Granulibacter* organisms to metabolize methanol, such as MDH and formaldehyde-activating enzyme [21]. These proteins had not been described as immunogenic in humans and may provide specific diagnostic targets for this organism. While patient sera detected the highly conserved bacterial proteins GroEL,

GroES, DnaK, and elongation factor TU, these have been detected in patients with *Helicobacter pylori* infection, as well as in patients with mycobacterial infections [22–25].

Approximately 45% of CGD patients showed immunoblot patterns indistinguishable from those seen for patients with culture-confirmed infection. It is surprising that almost 25% of healthy blood donors had identical patterns. While the number of seropositive specimens dropped significantly in the MDH antibody ELISA, rates among CGD patients continued to exceed rates among controls. The difference in seropositive rates between the immunoblot approach and the ELISA likely represents differences in both the targets and cutoffs used for defining positivity. To avoid having false-negative findings in our immunoblots, we screened individuals at a 1:250 dilution. This likely included sera that, if titered by immunoblot, would have rapidly lost signal with greater dilution. On the other

hand, our ELISA focused only on 2 *Granulibacter*-specific antigens, MDH1 and MDH2. Further refinement of our ELISA to improve sensitivity will allow us to better dissect seropositivity in CGD and non-CGD populations.

In both CGD and healthy patients, seropositivity might be explained by the following 3 possibilities: subclinical and self-limited infections, inadvertently treated infections, or frequent reexposure. In CGD patients, the majority of infections have not led to death. In addition, both healthy and CGD mice have had relatively benign infection courses despite persistent *G. bethesdensis* infection over several months [7]. In contrast, other CGD pathogens, such as *Burkholderia*, *Chromobacterium*, and *Aspergillus* organisms, cause significant morbidity and mortality in both humans and mouse models of CGD [26, 27].

The 5 CGD patients with reactive MDH ELISAs from whom *Granulibacter* organisms had never been isolated may have had past or persistent *Granulibacter* infection. The ability of *Granulibacter* organisms to persist and relapse after years of clinical stability [10] suggests that *Granulibacter* organisms are among the select organisms, at least in CGD, that can establish persistence and possibly latency. In tuberculosis, immune-based assays poorly discriminate prior from active disease and poorly predict reactivation [28]. These latency-establishing infections cause mild or unapparent disease in the overwhelming majority of otherwise healthy hosts but can vex immunocompromised patients with severe invasive or destructive disease.

A serodiagnostic test might identify patients with quiescent infection who are at risk for reactivation. The strongly positive ELISA signal in patient 3 years before *Granulibacter* organisms were recovered during an acute illness indicates that correlating MDH seroreactivity with acute infection will be difficult. In addition, this patient had repeated infection with genetically distinct strains of *Granulibacter* organisms. Whether this prolonged seropositivity reflected spontaneous resolution or inadvertent treatment of *Granulibacter* infection followed by reinfection is unknown [10]. However, patient 5 had a clinical and laboratory presentation compatible with acute *Granulibacter* infection, and his culture positivity was associated with the acquisition of high-titer antibody.

Microbiologic recovery of *Granulibacter* organisms from clinical specimens is difficult, suggesting that the frequency of active infection is likely to be underestimated. The absence of correlation between antibody titer and severity of illness in patients with culture-positive infection is reminiscent of other conditions, such as cystic fibrosis with infection due to *B. cepacia* complex or *Pseudomonas aeruginosa* [29]. Frequent testing of our patients with culture-confirmed infection will be needed to fully address whether changes in antibody titer over time have any relationship to treatment response or failure. These seroprevalence tools, a highly sensitive immunoblot and a simple and specific ELISA, will allow us to better

understand this emerging infection that appears to be affecting significant numbers of CGD patients and, possibly, healthy individuals alike.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online (<http://jid.oxfordjournals.org/>). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

Notes

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Potential conflicts of interest. D. E. G. and S. M. H. have a patent pending on the use of *G. bethesdensis* for industrial purposes. All other authors report no potential conflicts.

All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed.

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