Profiling the Humoral Immune Response of Acute and Chronic Q Fever by Protein Microarray*

Adam Vigil†‡, Chen Chen§, Aarti Jain‡, Rie Nakajima-Sasaki‡, Algimantas Jasinskas‡, Jozelyn Pablo‡, Laura R. Hendrix§, James E. Samuel§, and Philip L. Felgner‡

Antigen profiling using comprehensive protein microarrays is a powerful tool for characterizing the humoral immune response to infectious pathogens. *Coxiella burnetii* is a CDC category B bioterrorist infectious agent with worldwide distribution. In order to assess the antibody repertoire of acute and chronic Q fever patients we have constructed a protein microarray containing 93% of the proteome of *Coxiella burnetii*, the causative agent of Q fever. Here we report the profile of the IgG and IgM seroreactivity in 25 acute Q fever patients in longitudinal samples. We found that both early and late time points of infection have a very consistent repertoire of IgM and IgG response, with a limited number of proteins undergoing increasing or decreasing seroreactivity. We also probed a large collection of acute and chronic Q fever patient samples and identified serological markers that can differentiate between the two disease states. In this comparative analysis we confirmed the identity of numerous IgG biomarkers of acute infection, identified novel IgG biomarkers for acute and chronic infections, and probed for the first time the IgM antibody repertoire for both acute and chronic Q fever. Using these results we were able to devise a test that can distinguish acute from chronic Q fever. These results also provide a unique perspective on isotype switch and demonstrate the utility of protein microarrays for simultaneously examining the dynamic humoral immune response against thousands of proteins from a large number of patients. The results presented here identify novel seroreactive antigens for the development of recombinant protein-based diagnostics and subunit vaccines, and provide insight into the development of the antibody response. *Molecular & Cellular Proteomics* 10: 10.1074/mcp.M110.006304, 1–12, 2011.

*Coxiella burnetii* is an obligate intracellular bacterium and etiological agent of Q fever. *C. burnetii*'s remarkable stability, aerosol transmission, and infectious dose approaching one organism, have all contributed to its classification as a CDC category B bioterrorist agent. *C. burnetii* is widely distributed throughout the world (with the exception of New Zealand) and infects a variety of mammals, birds, reptiles, fish, and ticks (1). Naturally infected animals rarely demonstrate disease except for reproductive disorders, including late term abortion (2). Although the majority of infection in humans may be asymptomatic or manifest as a self-limiting mild disease, acute Q fever causes disabling influenza-like illness and may lead to death (3). Often misdiagnosed, the disease presents in the acute form as flulike illness with fever, chills, malaise, and a characteristic periorbital pain (4). Chronic infection is uncommon, but is a much more serious disease. As the result of endocarditis after acute Q fever is estimated to be up to 40%, follow-up IgG serology is suggested to be done at 3 and 6 months after acute disease to obtain earlier diagnosis of chronic infection. Diagnosis of the disease and assessment of incidence is hindered by variable clinical manifestations and often diagnosed only if it has been systematically considered. Q fever diagnosis relies upon serological methods and are available only in reference laboratories. IFA, and to a lesser extent ELISAs, are the predominant reference diagnostics and recognize IgG, IgM, and IgA reactivity to Phase I and Phase II organisms. Because cultivation of *C. burnetii* is difficult, hazardous, and requires special equipment, the antigens cannot be prepared in most clinical laboratories, and therefore are of limited use for routine diagnosis and large-scale investigations. The need for both standardization of diagnostics antigens and characterization of the seroreactive antigens of *C. burnetii* provide a strong rationale for comprehensive investigation. In this study we have utilized an emerging and powerful approach (5–12) to profile the antibody repertoire generated in response to infection using protein microarrays. Detection of an IgM immune response is important for early diagnosis of infectious disease. IgM antibodies appear early in the course of infection prior to class switch recombination (CSR)¹. B cells that undergo CSR do so in order to elicit a

¹The abbreviations used are: CSR, class switch recombination; ORF, open reading frame; IVTT, in vitro transcription/translation reactions; HA, hemagglutinin; HIS, histidine; COG, Clusters of Orthologous Groups.
more effective immune response to infection. Investigation of these early antibodies, as well as other isotypes, may be important for early diagnosis of infection. Isotype switching mediates different physiological effects including opsonization, cell lysis, and degranulation of mast cells, basophils and eosinophils. Moreover, development of IgG antibodies is dependent on prior IgM production. Therefore, by determining antigen-specific IgM antibodies and subsequent IgG antibodies, one may be able to determine when an individual was exposed to a pathogen, and potentially the progression of infection and therapeutic response.

EXPERIMENTAL PROCEDURES

Patient Serum Samples—Human sera from Q fever patients were collected from Texas A&M under protocols approved and created by the Texas A&M Institutional Review Board. Sera from healthy individuals obtained from volunteers at the General Clinical Research Center at the University of California, Irvine. Written, informed consent was obtained from participants. Acute Q fever patients and chronic Q fever patients were selected based on the long time (over 6 months) sera surveillance with the supportive clinical symptoms. Q fever IFA responses were determined with Q fever IFA IgG Kit (FOCUS Diagnostic, Cypress, CA) according to manufacturer’s instructions. Patients had elevated antiphase II IgG antibodies (IFA=1:64) at early time post-onset of fever followed by decreased antiphase II IgG titer without significant increase in antiphase I IgG antibodies were classified as acute Q fever patients. For antibody class switching study, 25 paired (“early” and “late”) sera samples from 25 acute Q fever patients were selected. The “early” sera were collected between 2–4 weeks post-onset of fever, and “late” sera were collected 2 weeks later. For profiling the IgG and IgM repertoire study, 96 acute/convalescent sera (>6 weeks post-onset of fever) were selected. For acute and chronic comparison study, thirty sera samples from chronic Q fever patients were selected based on the presentation of endocarditis (22/30) or other diverse (hepatitis, chronic fever) and persistent (>3 months) clinical presentation (8/30) accompanying with persistent high antiphase I IgG titer (IFA=1:128). Eighteen geographically matched, IFA-negative human sera and 36 internal health control sera were selected from our human serum library. Healthy control patient sera samples were collected under standard operating procedures with approval from the General Clinical Research Center at the University of California, Irvine (IBC#2003–1082). Sera samples were collected without anti-coagulants or other additives.

PCR Amplification and High-Throughput Recombination Cloning of C. burnetii ORFs—A detailed description of a majority of the cloning was previously described elsewhere (13, 14). In brief, the proteome of C. burnetii strain RSA 493 was cloned using a high-throughput PCR recombination cloning method developed in our laboratory (15). All 2084 open reading frames (ORFs) of a revised C. burnetii genome sequence (AE016828) and 105 unique ORFs from CbUK Q154 (CP001020) and CbUg Q212 (CP001019) larger than 50 amino acids in length were attempted to be cloned into pXT7 vector using a high-throughput PCR recombination cloning method previously described (15). Four ORFs (CBU0231, CBU0388, CBU0788, CBU1226, and CBU1427) were split into three segments because of size. ORFs were cloned using 20 bp ORF sequence-specific PCR primers to the 5’ and 3’ ORF ends. A unique 20 bp homologous recombination “adapter” sequence was included at the end of the 5’ and 3’ ORF specific primers (ACGACAAGCATATGCTCGAG and TCCGGAACATCGTATGGGT AA respectively). The adapter sequences, which become incorporated into the termini flanking the amplified gene, are homologous to the cloning sites of the linearized T7 expression vector pXT7. Following the manufacturer’s protocol, PCR reactions were prepared using Invitrogen Accuprime Taq Polymerase with 2.5 ng of template. Cycling conditions were as follows: 94 °C for 2 min; 30 cycles of 94 °C for 0.33 min, 55 °C for 0.25 min, 50 °C for 0.25 min, 68 °C at 1min/kb; and a final extension of 68 °C for 10 min. All ORF-PCR reactions were confirmed by gel electrophoresis for correct insert size prior to cloning into pXT7. pXT7 plasmid (3.2kb, KanR) encodes an N-terminal 10 x histidine (His) tag and a C-terminal hemagglutinin (HA) tag. Linearized pXT7 was diluted to 10 ng/μl, mixed with 1 μl of ORF PCR reaction mixture at a volume ratio of 4:1, and incubated on ice for 2 min, followed by addition of 10 μl of competent DH5α cells (Mc Labs). Reactions were mixed, incubated on ice for 30 min, heat shocked at 42 °C for 1 min, and chilled on ice for 1 min. 200 μl of SOC media was added and cells were incubated for 1 h at 37 °C. The entire reaction mixture was added to 1 ml of LB medium supplemented with 50 μg/ml kanamycin, and incubated overnight at 37 °C with shaking. Plasmids were isolated using QiAprep 96 Turbo kits (Qiagen, Valencia, CA) without colony selection. Minipreps of all attempted clones were analyzed by agarose gel electrophoresis to confirm insert size. Of the 2201 target ORFs/segments, 2087 were successfully cloned, giving a cloning efficiency of 94%. All clones were verified for presence of insert by gel electrophoresis. 955 clones were confirmed to be cloned insert by PCR-amplification using ORF-specific primers. Twenty-five percent of the cloned ORFs were selected at random and sequenced in both directions to verify target sequence match, and orientation, and checked for mutations introduced in the overlapping region during homologous recombination. In >99% of the sequences, the correct insert was verified.

Protein Microarray Chip Printing—The expression of cloned ORFs was performed in five hour in vitro transcription-translation (IVT) reactions (RTS 100 kits from Roche) according to the manufacturer’s instructions. Unpurified IVT reactions were printed onto nitrocellulose coated glass FAST slides (Whatman) using an Omni Grid 100 microarray printer (Genomic Solutions). 3.3 μl of 0.2% Tween-20 was mixed with 10 μl of IVT reaction and transferred to 384-well plates. Plates were centrifuged at 1600 × g to pellet any precipitate and remove air bubbles prior to printing. Supernatants were printed immediately without purification, and all ORFs were spotted in duplicate. Data values reported herein represent an average of the pair, unless otherwise mentioned. In addition, each chip was printed with control spots consisting of IVT reactions without the addition of a plasmid, purified IgGs, and purified EBNA1 proteins. Protein expression was confirmed by using monoclonal antipolyhistidine (clone His-1, Sigma-Aldrich, St. Louis, MO) and anti-hemagglutinin (clone 3F10, Roche) against the respective tags. Anti-His and anti-HA antibodies are conjugated to biotin and bound antibodies are detected by incubation with streptavidin-conjugated SureLight® P-3 (Columbia Biosciences). Intensities are quantified using QuantArray software package. All signal intensities are automatically corrected for spot-specific background.

Microarray Probing—Serum samples were preabsorbed with Escherichia coli lysate prior to array staining to remove background reactivity to E. coli proteins in the IVT reactions. The sera were diluted to 1/200 in Protein Array Blocking Buffer (Whatman) containing E. coli lysate at a final concentration of 30% v/v and incubated at room temperature for 30 min with constant mixing. The arrays were rehydrated in blocking buffer for 30 min and probed with the preabsorbed sera overnight at 4 °C with constant agitation. The slides were then washed five times in 10 ml Tris (hydroxymethyl) aminomethane buffer (pH 8.0) containing 0.05% (v/v) Tween-20 (TBBS), and incubated in biotin-conjugated goat anti-human IgG (anti-IgGfcγ, Jackson Immuno Research), or goat antihuman IgM, (anti-Fcγμ, Jackson Immuno Research) diluted 1/200 in blocking buffer. After washing the slides three times in TBBS, bound antibodies were detected by incu-
bation with streptavidin-conjugated SureLight® P-3 (California Biosciences). The slides were then washed three times in TTBS and three times in Tris buffer without Tween-20 followed by a final water wash. The slides were air dried after brief centrifugation and analyzed using a Perkin Elmer ScanArray Express HT microarray scanner.

**Immunostrip Probing and Development**—Sequence confirmed plasmids were used for expression in five-hour IVTT reactions, according to the manufacturer’s instructions. Unpurified proteins from the IVTT reactions were printed on Optitran BA-S 85 0.45 μm Nitrocellulose membrane (Whatman) using a BioJet dispenser (BioDot) at 1 μl/cm, and cut into 3 mm strips. Individual strips were then blocked for 30 min in 20% nonfat dry milk dissolved in TTBS. Prior to immunostrip probing, sera were diluted 1:250 in 20% nonfat dry milk solution containing 15 mg/ml E. coli lysate, and incubated for 30 min with constant mixing at room temperature. Preabsorbed sera were then applied to each strip and incubated overnight at 4 °C with gentle mixing. Strips were washed five times in TTBS, and then incubated for 1 h at room temperature in alkaline phosphatase conjugated goat anti-human immunoglobulin (anti-IgG, Fcγ fragment-specific; Jackson Immunoresearch, West Grove, PA) that was diluted to 1:5000 in TTBS. The strips were then washed three times in TTBS, followed by another three washes in Tris buffer without Tween-20. Reactive bands were visualized by incubating with 1-step Nitro-Blue Tetrizolium Chloride/5-Bromo-4-Chloro-3′-Indolyphosphate p-Toluidine Salt (NBT/BCIP) developing buffer (Thermo Fisher Scientific) for 2.5 min at room temperature. The enzymatic reaction was stopped by washing the strips with tap water. Strips were air dried and scanned at 2,400 dpi (Hewlett-Packard scanner). Images were converted to gray scale format by Photoshop and unaltered images are shown. Band intensities were quantified using ImageJ software (16) (found at http://rsbweb.nih.gov/ij/).

**Data and Statistical Analysis**—Microarray spot intensities were quantified using QuanArray software utilizing automatic background subtraction for each spot. Proteins were considered to be expressed if either tag’s signal intensity was greater than the average signal intensity of the IVTT reaction without plasmid, plus 2.5-times the standard deviation. “No DNA” controls consisting of IVTT reactions without addition of plasmid were averaged and used to subtract background reactivity from the unmanipulated raw data. Results herein are expressed as signal intensity. As previously reported (17), utilization of the “vsn” package found in the Bioconductor suite (http://bioconductor.org/) in the R statistical environment (http://www.R-project.org) was used to calculate p values. Specifically, differential analysis of the normalized signals was performed using a Bayes-regularized t test adapted from Cyber-T for protein arrays (18–21).

**RESULTS**

**Construction and Profiling IgG and IgM Seroreactivity in Longitudinal Samples**—C. burnetii ORFs cloned into the pXT7 vector were expressed under the T7 promoter in a 5 h cell-free IVTT reaction according to manufacturer’s instructions (see Methods). IVTT Expression efficiency was determined by probing against the amino-terminal His and carboxy-terminal HA tags for each spot (supplemental Fig. S1A). Proteins were considered to be expressed if either the HA or His tag signal intensity was greater than the average No DNA signal intensity plus 2.5 times the standard deviation; resulting in 97.8% of the C. burnetii ORFs considered positively expressed. In this manner a protein microarray comprised of 2001 expression-confirmed proteins, consisting of 2000 ORFs of C. burnetii strain RSA 493, with positive and negative controls. One ORF (CBU0231) was split in two because of its large size. In order to determine the dynamics of the antibody profile in acute Q fever we probed the full-proteome microarray with longitudinal samples of acute Q fever positive sera from 25 patients and 18 healthy naïve patient samples for both IgG and IgM seroreactivity. The fifty acute Q fever sera samples were collected from 25 patients at two time points referred to as “early” and “late.” Early and late longitudinal samples were collected 18 ± 4 days between time points. Representative microarray images are shown in supplemental Fig. S1B. Signal intensities were quantified and the seroreactivity for each antigen was recorded for each patient individually for both IgG and IgM. As such the total IgG antibody response to C. burnetii of the longitudinal sera collection was determined to seroreact with 49 antigens or 2.5% of all of the antigens printed on the array. The total IgM antibody response of the sera collection was determined to seroreact with 261 antigens or 13.1% of all of the antigens printed on the array. Eighty-seven of the 261 IgM seroreactive antigens were highly seroreactive, having seroreactivity greater than six standard deviations above the NoDNA control. In total we found 113 ORFs that are highly IgM seroreactive and IgG seroreactive.

**Limited Changes in the Average Seroreactivity Between Early and Late Time Points of Acute Infection**—Average seroreactivity of all 113 antigens was compared between early and late time points for acute infection. Individual antigen antibody seroreactivity was more consistent between early and late time points for IgG than IgM (Fig. 1) with an R squared of 0.90 and 0.85, respectively. Importantly, no antigens were considered differentially reactive between early and late time points for either IgG or IgM (two tailed Student’s t test p value <0.05). A bar graph of average signal intensity for all 113 antigens illustrates high levels of consistency between early and late time points (supplemental Fig. S2A). Rate of change of signal intensity comparison between time points measured by linear regression of the 113 antigens indicate a small decrease in IgM antibody seroreactivity (slope of 0.92 and 1.05 for IgM and IgG, respectively). Additionally, we found that average seroreactivity is consistent despite the varied breadth and magnitude of the IgG and IgM responses (Fig. 1 and supplemental Fig. S2B).

**Diverse Individual Humoral Immune Response**—Overall, the average IgG and IgM signal intensity was consistent between early and late time points. However when comparing seroreactivity on an individual patient basis we found more variation. A heatmap of IgG and IgM seroreactivity for each patient is shown in Fig. 2. Patient samples are sorted left to right by increasing seroreactivity to all IgG or IgM reactive antigens and are in the same order for early and late time points for visual comparison. Here we found the total IgG or IgM response was variable between the 25 patients. Furthermore, individual patient IgG and IgM seroreactive intensities were totaled and compared in supplemental Fig. S3. Ten of the
patients had higher IgM than IgG antibody responses, 11 had approximately equal levels, and 4 had higher IgG than IgM seroreactivity. Also the range of IgG and IgM response was highly variable, with some patients having significant levels of IgG, IgM, or both. Individual signal intensity change was calculated between early and late time points for IgG/M and shown as an orange to blue heat map. We found frequent changes for IgG and IgM on an individual basis (Fig. 2). IgM signals had more frequent changes of >2000 signal intensity units than IgG. Several antigens with significant decreases in IgM seroreactivity had corresponding increases of IgG seroreactivity. Altogether we found a diversity in the magnitude of the antibody profile for both IgG and IgM isotypes in acute infection with limited seroreactivity changes between two time points early in infection.

Identification of IgG Seroreactive Antigens in Acute and Chronic Fever Patients—We next profiled the antibody repertoire of a large sera collection in order to identify differentially reactive proteins between healthy, acute, and chronic Q fever patients. Three sets of pooled chronic patient samples were
probed for IgG and IgM seroreactivity on the full proteome microarray (\(n = 3\) patient samples per pooled sample). All proteins with individual patient seroreactivity greater than 2.5 times the standard deviation of the IVTT reaction without plasmid were considered reactive and were included on a down-selected microarray containing the previously identified reactive IgG and highly reactive IgM antigens, in addition to 68 (of 106) newly cloned antigens, for a total of 322 proteins. The 106 newly cloned antigens are unique ORFs and come from endocarditis patient isolates. These antigens are indicated with a “G” or “K” in the locus tag (e.g. CBUK1974). As such, we used the down-selected microarray to profile the IgG and IgM antibody repertoire of a large collection of patient samples from healthy and acute or chronic Q fever patients (\(n = 324\) samples). The down-selected microarray was probed with anti-His and anti-HA antibodies to determine protein expression efficiency. Protein expression efficiency of the down-selected microarray was determined to be 99.6%.

Ninety-six acute and thirty chronic Q fever patient, and thirty-six healthy control serum samples were probed on the down-selected microarray for both IgG and IgM. Representative chip images are shown in Fig. 3. Individual protein seroreactivity was recorded for all serum samples. Antigens were considered seroreactive if the average signal intensity exceeded the average signal intensity of the IVTT reaction without plasmid (No DNA controls) plus 2.5-times the standard deviation. In this manner, 156 antigens were seroreactive for either IgG or IgM in either acute or chronically infected patients. In total, 115 ORFs were IgG seroreactive for either acute or chronic infection, respectively. Importantly we found chronic Q fever patients exhibited an increase in the magnitude and breadth of the IgG antibody repertoire as shown in Fig. 4A. Only two antigens (CBU0733 and CBU1596) were seroreactive in acute Q fever patients that were not seroreactive in chronic infection patients. Although these two proteins met the criteria for seroreactivity in acute Q fever patients, they were not highly seroreactive and this low level of seroreactivity may explain why they did not also meet the criteria in chronic infection patients. Average seroreactivity for the 50 most seroreactive proteins of acute and chronic infection are displayed as a bar graph in Fig. 4B. In comparison to healthy individuals, 25 and 64 antigens were differentially reactive for acute or chronic Q fever patient samples, respectively. In general chronic infection patients had more extensive IgG seroreactivity, with the majority of the differentially reactive antigens of chronic patients overlapping with those of acute patients. All but two (CBU1719 and CBU0433) of the 25 antigens that were differentially reactive between healthy individuals and acute Q fever patients were also differentially reactive between healthy individuals and chronic Q fever patients. The remaining 41 antigens are uniquely differentially reactive between healthy individuals and chronic Q fever patients.

We next compared serological profiles between chronic and acute Q fever patients in order to identify IgG differential seroreactivity. As shown in Fig. 5, 26 antigens were IgG differentially seroreactive, and 129 were equally seroreactive. Of these 26 differentially reactive antigens, 5 and 24 were also differentially reactive when compared with healthy controls for acute and chronic infection patients. Only four antigens that were uniquely differentially reactive in acute samples compared with healthy controls were not also differentially reactive in chronic infection patients (CBU1719, CBU0433, CBU1920, and CBU0902). A histogram comparing all 26 differentially seroreactive antigens, and 26 cross-reactive antigens is shown in Fig. 5B.

Identification of IgM Seroreactive Antigens in Acute and Chronic Fever Patients—We next profiled the IgM antibody
repertoire of acute and chronic infection samples on the down-selected microarray. Here we found an inverse of seroreactivity profiles, with acute patients having more seroreactivity in terms of magnitude and breath. One hundred and fifteen and 62 antigens were IgM seroreactive for acute and chronic infection, respectively. Only three of the 62 IgM seroreactive antigens were uniquely seroreactive for chronically infected patients (CBU0443, CBUK0188, and CBU1430). Relative seroreactivity of all 118 IgM seroreactive antigens is displayed in Fig. 6 as a heat map. In comparison to naïve controls, 100 and 46 antigens were differentially reactive for acute and chronic infection patients, respectively. Of the 46 differentially reactive antigens for chronic infection patients, only two were not differentially reactive for acute infection patients (CBU1430 and CBU0443). Sixteen of the 118 seroreactive antigens were not differentially reactive for either acute or chronic infection patients and were considered cross-reactive. A histogram showing the seroreactivity of the twenty most seroreactive IgM and differentially reactive antigens for acute infection in comparison to naïve controls is shown in Fig. 6B. When we compared the seroreactive profiles between acute and chronic Q fever patients, we did not find any antigens that were significantly differentially reactive.

Probing Immunostrips for IgG Seroreactivity with Acute and Chronic Q Fever Patient Sera—Twenty seroreactive antigens that were either unique or in common between acute and chronic Q fever patients were printed onto nitrocellulose membranes, referred to as immunostrips. Individual immunostrips were probed for IgG antibodies with all 30 chronic infection patient sera, 12 acute infection patient sera chosen at random, and 7 healthy controls (Fig. 7). Chronic infection patient sera reacted strongly with several antigens, although the intensity pattern varied depending on the individual. Acute infection patients had weaker reactivity, and also exhibited a varied pattern of antigen recognition. Healthy controls had limited or no reactivity to \textit{C. burnetii} proteins, as expected, and their lack of seroreactivity is in agreement with the microarray results. Signal intensities were quantified as described in methods and compared between acute and chronic infection. CBU1910, CBU1863, CBU1718, CBU1627, and CBU1974 were all significantly lower in acute infection than chronic, and exhibited observable differences in seroreactivity pattern and intensity (p value $= 9.0 \times 10^{-3}, 1.2 \times 10^{-2}, 4.9 \times 10^{-2}, 2.9 \times 10^{-2}$, and $3.3 \times 10^{-3}$, respectively).

Identification of Potential Immunoglobulin Class-switching—We next screened for the hallmarks of class-switching from IgM to IgG. As such we looked for a decrease in IgM seroreactivity with an accompanying increase in IgG. Here we consider a potential isotype switch event as a decrease in IgM seroreactivity of 2000 signal intensity units followed by an increase in IgG of 2000 signal intensity units. When screening all 113 seroreactive antigens for switch events from the long-
Growth longitudinal patient sample set with acute Q fever we found 120 potential switch events or 4.8/1000. When comparing the average seroreactivity of these antigens we did not observe the canonical switch model. For example, as seen in supplemental Fig. S4, seven antigens had an increase in the average IgG signal intensity that was unaccompanied by changes in IgM, and two antigens had an increase in IgG and a small increase in IgM. We also found unpaired changes of IgM seroreactivity for nine antigens with increasing, and nine antigens with decreasing average IgM seroreactivity. When screening against all of the antigens on the microarray we found that the average number of switch events was 19.64/1000 per patient. This indicates a large percentage of potential switch events occur throughout the proteome early in infection. The largest number of switch events for a single antigen was observed for CBU1718 (GroEL) with seven patients exhibiting potential switch. The next highest number of switch events occurred with CBU545, CBU1627, CBU0494, CBU1867, CBU1997, and CBU0510c with four individuals exhibiting switch events. This small frequency of switch events may be because of the limited duration of time between time points.

We next screened for switch events using the average seroreactivity profiles of acute and chronic Q fever patients probed against the down-selected microarray. As shown in supplemental Fig. S5, we identified ten antigens that conformed to the canonical model (CBU1718, CbuK1974, CBU1910, CBU0092, CBU0545, CBU0109, CBU2029, CBU1810, CBU1586, and CBU0753). We again observed numerous examples of uncoupled increases in IgG and uncoupled decreases in IgM seroreactivity. In total we found 31 antigens with an increase in IgG response and no changes in IgM response. Conversely 15 antigens exhibited a decrease in IgM response that was uncoupled to changes in the IgG response. Unlike in the longitudinal sample comparison of acute infection we did not observe many instances of decreases in average IgG signal, nor increases in IgM signal. Uncoupled changes may be indicative of a memory response in B cells specific for these proteins or homologs of these proteins, or antigen expression loss. Further examination of switch events in animal models are necessary to address this observation.

**Functional Classification of IgM and IgG Seroreactive and Differentially Reactive Antigens**—The identification of IgG and IgM antibodies in the longitudinal sample collection was similar to the profile we determined for acute and chronic infection. All ten of the most reactive antigens identified by IgM were identified on the down-selected array. Nine of the top 10
IgG seroreactive antigens were also identified in the full-proteome array. The missing antigen, CBUK1974, but was not printed on the full-proteome array. As such we combined all of the identified seroreactive antigens from both the down-selected and full proteome microarray for both acute and chronic infection, at early and late time points. In total we found 343 seroreactive ORFs, of which 130 were IgG seroreactive antigens and 307 were IgM seroreactive antigens (listed in supplemental Table S1). 94 antigens were seroreactive in both IgG and IgM antigen sets, 36 antigens were specific for IgG, and 213 were specific for IgM. A comparison of computationally predicted features of all seroreactive antigens reveals that the immune response is not evenly distributed (supplemental Table S2). We found significant enrichment of...

Fig. 6. IgM antibody profile comparison of healthy patients to acute and chronic Q fever patients. A, Heatmap showing normalized intensity with red strongest, green weakest, and black in between. Patient samples are in columns and sorted left to right by increasing average intensity to differentially reactive antigens, and antigens in rows sorted by decreasing average seroreactivity of acute patients. Differentially and cross-reactive antigens are separated. Only seroreactive antigens are displayed. B, The mean seroreactivity of each antigen was compared between healthy patients and either acute or chronic Q fever patients. Corresponding p values for each antigen is shown as a green line on the secondary y axis.

Fig. 7. Immunostrip probing. Twenty differentially reactive antigens were printed onto nitrocellulose paper using a BioDot jet dispenser. Strips were probed with healthy, acute, and chronic Q fever patient sera followed by alkaline phosphatase-conjugated secondary antibody and enzyme substrate. Weak reactivity in the acute infection patients can be distinguished from the strong reactivity in chronic infection patients.
IgG and IgM seroreactivity for proteins with a single predicted transmembrane domain, or with a predicted signal peptide sequence. Conversely, proteins were significantly less likely to be seroreactive for IgG or IgM if they contained no predicted transmembrane domains, or did not contain a predicted signal peptide sequence. These trends were also the same for the subset of proteins that were IgG or IgM differentially reactive. Proteins with five or more predicted transmembrane domains were also significantly less likely to be IgG or IgM seroreactive. Interestingly we did not find significant enrichment for proteins based on cellular localization predicted by PSORTb, with the exception of unknown PSORTb category (1.11 fold enrichment, \( p = 4.2 \times 10^{-5} \)). Proteins with “unknown” PSORTb cellular location were also significantly more likely to be seroreactive for only IgM seroreactive proteins. Although not statistically significant, PSORTb predicted outer membrane proteins were enriched for both IgG and IgM (2.47 fold enrichment, \( p = 1.2 \times 10^{-2} \) and 1.04 fold enrichment, \( p = 1 \times 10^{-6} \), respectively). In comparison, we have previously found PSORTb predicted outer membrane proteins to be significantly enriched for *Burkholderia pseudomallei* (17) and *Bartonella henselae* (22).

Utilization of the NCBI database of Clusters of Orthologous Groups (COGs) of proteins allowed us to functionally characterize the seroreactive antigens and identify groups that were either over- or underrepresented. Each COG consists of individual proteins or groups of paralogs from at least three lineages, and is comprised of 25 categories of functional definitions. Each protein in the database is assigned to one or more COGs with a total of 1757 assigned COG annotations and 485 ORFs not listed in the COG database. ORFs that were not in the COG database were significantly more likely to be IgM seroreactive (1.23 fold enrichment, \( p = 1.72 \times 10^{-5} \)), COG E (Amino acid transport and metabolism) and COG G (Carbohydrate transport and metabolism) were both significantly less likely to be IgG and IgM seroreactive. None of the 85 COG H (Coenzyme transport and metabolism) proteins were IgG seroreactive. Conversely, COG I (Lipid transport and metabolism) was significantly more likely to be IgG seroreactive (6.72-fold enrichment, \( p = 1.2 \times 10^{-4} \)). Proteins categorized as (Cell wall and membrane biogenesis) COG M, were significantly more likely to be IgG differentially reactive. COGs U (Intracellular trafficking and secretion), and COG V (Defense mechanisms) were also significantly more likely to be IgG and IgM differentially reactive. Both of these COG functional categories were also significantly more likely to be IgG differentially reactive for *Bartonella henselae* infection (22).

**DISCUSSION**

Here we profiled the antibody response to *C. burnetii* infection in a well-defined longitudinal sera collection from acute infections and in a large collection of patient sera of acute or chronic infection. We found that antigens that bind to IgG antibodies are very limited for acute infection (3.1% of the antigens on the array), whereas the IgM response is fivefold more extensive and seroreacts with 13.4% of the antigens in the proteome. Conversely, the IgG reactivity during chronic infection was broader and reacted with 6.2% of antigens within the proteome, yet exhibited a narrower and weaker IgM seroreactivity profile in the chronic Q fever patients’ samples. Chronic Q fever patients had IgM seroreactivity to only 62 antigens, compared with 115 in the acute Q fever patients. Although the more extensive repertoire of IgM responses in acute samples was to be expected, the results here provide a more informative determination of the extent, and importantly the identity of both IgG and IgM seroreactive antigens.

In this study we determined the IgG and IgM profile of acute *C. burnetii* infection between two time points in order to observe the dynamics of antibody response during infection. We found that average seroreactivity was highly similar between early and late time points of acute infection. Moreover, on average, patients infected with *C. burnetii* undergo very limited changes in both the IgG and IgM response between early and late time points. The highly consistent IgG and IgM profiles between these time-points are encouraging for the development of diagnostics. However, many of the individual patient humoral immune responses had bias toward either IgG or IgM, and exhibited more diversity than the average repertoire. This variation in response for acute Q fever patients provide a rationale for development of diagnostics that detect both IgG and IgM seroreactive antigens. Furthermore, utilization of numerous proteins for diagnosis for both IgG and IgM would be necessary for accurately detecting infection from patients that exhibit diverse responses.

By using the antibody profiles for early and late acute Q fever, we could observe the dynamics of the antibody repertoire at a unique level not possible by other methods. Using these profiles we screened for changes in IgG and IgM profile early in infection that could indicate potential switch events. On an individual patient level, we observed numerous instances that suggest isotype switching. We found over the entire microarray that the average number of switch events was 19.64 ± 31. Surprisingly, we found that the fluctuation in IgG and IgM response between early and late time points was very minimal on a group level and only found four antigens that fit the classical isotype switch model. Interestingly, CBU1718 and CBU1719 (GroEL and GroES, respectively) had high levels of IgM reactivity and appeared to undergo isotype switch in the longitudinal samples. We have found that despite the reported cross-reactivity of GroEL and GroES, the increase in seroreactivity in acute infections of IgG (and possibly IgM) may still be utilized for differential diagnosis compared with healthy controls and unrelated bacteria. The degree of relevant cross-reactivity to related infectious agents, which present similar symptoms, still needs to be determined for accurate specificity. For example, we reported that *Burk-
Differential Antibody Repertoire in Q Fever Patients

holderia psuedomallei GroEL was still significantly differentially seroreactive in comparison to other cases of bacteremia (17, 22). The other two consistent switch events are antigens CBU0092 and CBU0545 (the YbgF tol-pal system protein and Lema proteins, respectively). CBU0092 (tol-pal system protein YbgF) was found in both phases of C. burnetii (23, 24) and experiments using E. coli mutants with defects in tol-pal proteins led to a loss of outer membrane integrity leading to hypersensitivity to drugs and detergents, periplasmic leakage, and outer membrane vesicle formation (25–27). We have consistently identified CBU0545 (lema protein) as a diagnostic antigen (13, 14) for acute Q fever. It contains a predicted transmembrane domain, and is part of the GacS/GacA system. The GacS/GacA system controls the production of extracellular enzymes involved in pathogenicity in animals, ecological fitness, or tolerance to stress. These proteins are consistently seroreactive and their role in the essential life cycle of C. burnetii may make them potential candidates for recombinant protein-based vaccination.

Recent studies indicated that antibodies plays an important role in protective immunity in several intracellular pathogens (28–30). Adoptive transfer of serum from vaccinated mice protects against C. burnetii challenge in naïve mice (29, 30), which suggests that antibodies also contribute to protective immunity against C. burnetii. In both our previous studies (13, 14) and here we have identified several seroreactive antigens, that may be important for protective immunity in infection (CBU1910, CBU0891, CBU0109, CBU1143, CBU0612, CBU0092, and CBU0545). In this study we used an expanded array containing K and G proteins and were able to identify CbuK1974 as a potentially important seroreactive protein in both acute and chronic infection. CbuK1974 nucleotide sequence is conserved in CbuK_Q154, CbuG_Q212, RSA 493, RSA 331, and Dugway 5J108–111. It does not appear to be found in other organisms, and its protein sequence loosely aligns with a putative lipoprotein in Clostridium perfringens ATCC 13124 (accession YP_695526.1, e = 9.9). CBUK1974 has unknown predicted cellular location by PSORTb, no predicted transmembrane domains, and contains a predicted signal peptide sequence. CBUK1974 was the second and fifth most IgG reactive antigen in chronic and acute infection patients, respectively. It was significantly more reactive in acute and chronic patients in comparison to healthy control samples, and is also differentially reactive between acute and chronic patients. It is a small protein (63 amino acids) that was found in the genome of C. burnetii strains isolated from endocarditis patients. Its unique characteristics and high seroreactivity warrant further characterization of its biological significance, and it may serve as an ideal potential candidate for diagnostics and vaccine development.

Antibody-mediated protection against Q fever may be because of different mechanisms, such as complement activation, antibody-dependent cell-mediated cytotoxicity (ADCC), bactericidal and neutralization function; all of which require a diverse antibody repertoire including specific antigen recognition and appropriate isotype-determined effector function. We extended the screen for hallmarks of isotype switching between the large sera collection of acute and chronic Q fever patients. In this screen we identified ten antigens that exhibited potential switch events. All ten of these antigens were differentially reactive in comparison to naïve controls and may be useful in recombinant protein-based diagnostic assays. Importantly we found a screen of acute and chronic patients that many antigens exhibited changes in IgG/M seroreactivity that did not meet our criteria for identifying isotype switching. These antigens exhibited uncoupled changes in IgG/M seroreactivity that would not be influenced by confounding factors observed in a acute phase screen. Some possible explanations are recall response, changes in antigenic sources, protein homology to related bacteria, or non-conventional switch kinetics. Development of B-cell activation and appropriate CSR is mediated by many receptors including the B-cell receptor, costimulatory molecules, cytokine receptors, and Toll-like receptors. Immunoglobulin class switching and affinity maturation of B cells requires cognate help from anti-specific CD4+ T cells through T-cell receptor (TCR), co-stimulatory molecule CD40, and cytokines (31, 32). Several studies have demonstrated that there are intricate linkages between B cells and T helper cells, and that proteins recognized by the humoral immune response are also targeted by cellular immune responses (33–35). By investigating both of these criteria, one may determine an effective humoral immune response to infection. More specifically, investigation of the IgG and IgM response to C. burnetii may provide a better understanding of the humoral and cellular mediated immune response to obligate intracellular bacterium. In addition, CSR is closely associated with antigen-specific T helper cells activity, as preferential CD4+ T cell help is required for B cell proliferation and Ig isotype switching. Several studies have demonstrated that there are intricate linkages between B cells and T helper cells, and that proteins recognized by the humoral immune response are also targeted by cellular immune responses (33–35). By identifying which proteins are seroreactive we may also be identifying which proteins are targeted by the cellular immune responses. Here we report for the first time a comprehensive analysis of the isotype switch on an individual patient level. Utilization of this technique for other infectious agents, or in model organisms, will likely provide important insight into the dynamics of CSR, B-cell activation, immune evasion, and the influence the natural antibody repertoire on infection outcome.

In total we identified 130 antigens or 6.2% of the 2130 ORFs in the genome that are larger than 50aa in length that were IgG seroreactive. Our findings were consistent with our previous results and between both sera collections, with thirteen of the top 14 antigens seroreactive in both the acute sample collection and the longitudinal sample collection. Only 15 of the 49 antigens identified in the longitudinal sample collection were not reactive in the 115 acute/chronic sera
collections, identifying a total of 130 seroreactive antigens. Additionally, we did not find a correlation of the anti-tag signal intensity with sera signal intensity. We previously reported the identification of 21 IgG seroreactive antigens for acute infection (14). All but the least reactive antigen (CBU0436) was identified again. The four newly identified differentially reactive proteins between healthy and acute Q fever patients are CBU1920, CBU1725, CBU1718, and CBUK1974. CBU1920 was previously reported to exhibit seroreactivity in some patients, but was considered “highly variable.” We previously reported 63 proteins exhibiting a highly variable antibody response that did not meet our criteria for seroreactivity. Although these proteins were highly seroreactive in some individuals, the average seroreactivity was below our threshold, and was categorized as “highly variable.” Of the previously reported highly variable antibody responses, 34 antigens were printed on the down-selected chip, and almost half (16) were identified here as seroreactive. The other three newly identified antigens are CBU1725, CBU1718, and CBUK1974.

The proteins identified here may be used for the development of much-needed diagnostic tools and vaccine development. Current diagnostics for Q fever are based on both IgG and IgM response and require whole cell antigens to be cultured under BLS3 conditions. These diagnostics suffer from a lack of uniformity and specificity. Furthermore, there is no satisfactory vaccine that can be administered without prior screening for immunity in populations at risk of potential exposure to the agent. Currently there is no licensed vaccine, although a commercial vaccine is approved in Australia. In addition, identification of seroreactive antigens that provide strong humoral response that are specific and sensitive to Q fever is needed and will aid in the development of a safe and effective vaccine and reliable serodiagnostic tests using recombinant proteins. We utilized the IgG seroreactivity results to produce a proof of concept diagnostic assay. We previously reported the utility of immunostrips for identification of acute infection. Here we further developed the immunostrips to differentiate between acute and chronic infection. Although differentiation of acute and chronic infection can be performed on microarrays, use of microarrays is limited in general practice and would be cost prohibitive worldwide. Our previous results and the findings presented here indicate that multiple antigens would be necessary to more accurately determine infectivity from the diversity of the antibody profile observed in Q fever patients. As such we utilized 20 antigens in a proof of concept diagnostic device and found reactivity was varied in both acute and chronic infections. Purification of the recombinant proteins will improve the diagnostic potential of immunostrips in comparison to the crude IVTT reactions that were used here. Additionally, utilization of the IgM differentially reactive antigens identified here will further strengthen differentiation between healthy, acute, and chronic Q fever patients.

We also found that reactivity was not evenly distributed across the proteome, and no annotated category was completely seroreactive. As expected, proteins predicted to contain a signal peptide or transmembrane domain, are significantly overrepresented in the seroreactive and differentially reactive antigen list. Similarly, utilization of the NCBI database of Clusters of Orthologous Groups (COGs) of proteins database allowed us to characterize the seroreactive antigens and we found proteins involved in intracellular trafficking (COG U) and defense mechanisms (COG V) were also enriching features. Although these results classifying antigenicity by protein localization are consistent with and expectations and our past results, they provide a quantitative and more informative understanding than previously reported (14, 17, 36). Moreover, identification of categories of protein annotation that are underrepresented is important for understanding immune evasion and B-cell response. Here we found COG functional categories E, G, H, and L identified proteins were less likely to be immunoreactive, whereas COGs M, U, S, and V contained proteins significantly more likely to be reactive.

In summary we report a comprehensive protein microarray for C. burnetii probed with a longitudinal sample collection of acute infection. We found that an individual’s antibody repertoire is highly conserved within 18 days for both IgG and IgM, with a limited number of antigens having significant seroreactivity changes. Additionally, we probed a down-selected microarray with a large collection of acute and chronic infection sera in order to determine differentially reactive antigen profiles. The results of the down-selected microarray have practical applications for the development of novel subunit vaccines and recombinant protein-based diagnostics. We have identified unique signatures for acute and chronic infection by microarray, and have transferred these results onto a more universal platform. The antigens identified here may provide the basis for subunit vaccine development and the development of much needed improvements in reliable diagnostics.

Acknowledgments—We would like to thank Christopher M. Hung for excellent technical assistance.

REFERENCES
1. Sawyer, L. A., Fishbein, D. B., and McDade, J. E. (1987) Q fever: current concepts. Rev. Infect. Dis. 9, 935–946
2. Baca, O. G., and Paretsky, D. (1983) Q fever and Coxiella burnetii: a model for host-parasite interactions. Microbiol. Rev. 47, 127–149
3. Maurin, M., and Raoult, D. (1999) Q fever. Clin. Microbiol. Rev. 12, 518–553
4. Raoult, D., and Marrie, T. (1995) Q fever. Clin. Infect. Dis. 20:489–495; quiz

To whom all correspondence will be sent: Department of Medicine, Division of Infectious Diseases, University of California, Irvine, CA 92697, Tel.: 949-824-7387 (office number); Fax: 949-824-9675; E-mail: vigila@uci.edu.
Differential Antibody Repertoire in Q Fever Patients

5. Chen, C. S., Sullivan, S., Anderson, T., Tan, A. C., Alex, P. J., Brant, S. R., Cuffari, C., Bayless, T. M., Talor, M. V., Burek, C. L., Wang, H., Li, R., Datta, L. W., Wu, Y., Winslow, R. L., Zhu, H., and Li, X. (2009) Identification of novel serological biomarkers for inflammatory bowel disease using Escherichia coli proteome chips. Mol. Cell. Proteomics 8, 1765–1774

6. Keasey, S. L., Schmid, K. E., Lee, M. S., Meegan, J., Tomas, P., Minto, M., Tikhonov, A. P., Schweitzer, B., and Ulrich, R. G. (2009) Extensive antibody cross-reactivity among infectious gram-negative bacteria revealed by proteome microarray analysis. Mol. Cell. Proteomics 8, 924–935

7. Gray, J. C., Corran, P. H., Mangia, E., Gaunt, M. W., Li, Q., Tetteh, K. K., Gaseitsiwe, S., Valentini, D., Mahdavifar, S., Magalhaes, I., Hoft, D. F., Prasad, S., and Felgner, P. L. (2007) Profiling the antibody immune response based on mechanisms of antibody-mediated protection against intracellular pathogens. Adv. Immunol. 91, 487–513

8. Casadevall, E., Gavili, M., Sturgis, J. N., and Lloube’s, R. (2000) Proton membrane pal proteins in Escherichia coli. J. Membrane Biol. 177, 547–552

9. Bernadac, A., Gavioli, M., Lazzaroni, J. C., Raina, S., and Lloube’s, R. (1998) The Tol membrane pal proteins of Escherichia coli and their involvement in the uptake of biomolecules and outer membrane stability. FEMS Microbiol. Lett. 177, 191–197

10. Prasad, S., Hirsch, B., Kamerer, D. B., and Curtin, H. (1991) Odontoma of the middle ear cleft. Am. J. Otol. 12, 452–454

11. McHeyzer-Williams, L. J., and McHeyzer-Williams, M. G. (2005) Antibody specific memory B cell development. Annu. Rev. Immunol. 23, 487–513

12. Richards, K. A., Chaves, F. A., Krafick, F. R., Topham, D. J., Lazarski, C. A., and Sant, A. J. (2007) Direct Ex Vivo Analyses of HLA-DR1 Transgenic Mice Reveal an Exceptionally Broad Pattern of Immunodominance in the Human HLA-DR1-Restricted CD4+ T-Cell Response to Influenza Virus Hemagglutinin. J. Virol. 81, 7608–7619

13. Arens, R., Wang, P., Sidney, J., Loewendr, A., Sette, A., Schoenberger, S. P., Peters, B., and Benedict, C. A. (2008) Cutting edge: murine cytomegalovirus induces a polyfunctional CD4+ T cell response. J. Immunol. 180, 6472–6476

14. Chen, C., Bouman, T. J., Beare, P. A., Mertens, K., Zhang, Q. G., Russell-Lodidge, K. E., Hogaboam, J. P., Peters, B., Felgner, P. L., Brown, W. C., Heinzen, R. A., Hendrix, L. R., and Samuel, J. E. (2009) A systematic approach to evaluate humoral and cellular immune responses to Coxiella burnetii immunoreactive antigens. Clin. Microbiol. Infect. 15, 156–157

15. Sette, A., Moutaftsi, M., Moyron-Quiroz, J., McCausland, M. M., Davies, D. H., Johnston, R. J., Peters, B., Rafii-El-Idrissi Benhnia, M., Hoffmann, J., Yu, H. P., Singh, K., Garboczi, D. N., Head, S., Grey, H., Felgner, P. L., and Crotty, S. (2008) Selective CD4+ T cell help for antibody responses to a large viral pathogen: deterministic linkage of specificities. Immunity 28, 847–858

16. Vigil, A., Davies, D. H., and Felgner, P. L. (2010) Defining the humoral immune response to infectious agents using high-density protein microarrays. Future Microbiol. 5, 241–251

In order to cite this article properly, please include all of the following information: Vigil, A., Chen, C., Jain, A., Nakajima-Sasaki, R., Jasinskas, A., Pablo, J., Hendrix, L. R., Samuel, J. E., and Felgner, P. L. (2011) Profiling the Humoral Immune Response of Acute and Chronic Q Fever by Protein Microarray. Mol. Cell. Proteomics 10(10):M110.006304. DOI: 10.1074/mcp.M110.006304.