Journal of Infection and Public Health (2012) 5, 321—331

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SHORT REPORT

Comparison of total antibody and interferon-γ T-cell responses in patients following infection with brucellosis in Georgia

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Received 19 July 2011; received in revised form 13 February 2012; accepted 22 March 2012

KEYWORDS
Brucella;
Immune response;
ELISPOT;
ELISA;
Brucellin;
LPS

Summary Brucellosis is an ancient disease that still remains a significant threat to humans and is typically linked to exposure to infected animals and/or consumption of unpasteurized animal products. Despite this history, we have a relatively limited understanding of the host characteristics of this disease; consequently, further research is necessary. In this study, we examined the humoral immune response in 43 Georgian individuals that had been diagnosed with brucellosis 3—12 months before enrollment in the study, many of whom still had symptoms after the completion of antibiotic therapy. In total, 35 of 43 (83%) of the patients had antibodies that bound to *Brucella* lipopolysaccharide (LPS) by COMPELISA, and 34 of 38 (89%) patients had demonstrable specific antibodies to Brucellergene™ antigens; the results from the two ELISAs were highly correlated ($p = 0.031$, $r = 0.851$). We also studied the cellular immune responses in 15 patients. All of the patients generated interferon (IFN)-γ in response to *ex vivo* stimulation with *Brucella* protein antigens, and the majority

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http://dx.doi.org/10.1016/j.jiph.2012.03.006

of the patients maintained measurable humoral responses to both LPS and protein antigens. From this initial study, we conclude that measurement of antibody and of cellular (IFN-γ) responses to brucellergene OCB protein epitopes may be worthy of further investigation as an alternative or adjunct to current diagnostics.
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Introduction

Brucellosis is a chronic, debilitating infection that is responsible for substantial morbidity worldwide [1]; it is caused by a variety of *Brucella* species, which are Gram-negative coccobacilli and intracellular pathogens. *Brucella* species are zoonotic, and infections occur in a wide variety of mammalian hosts, including domestic livestock, such as cattle, sheep, goats and pigs. Human disease is typically linked to exposure to infected animals (such as during slaughtering) or consumption of unpasteurized animal products, such as milk or cheese [2]. Patients often require prolonged antibiotic therapy, and relapses of infection can occur after therapy [3]. *Brucella* species are also considered to pose a substantial threat as possible biological weapons and are included on the CDC's list of possible bioterrorism threat agents [4]. Brucellosis is endemic in Georgia and causes approximately 150 registered cases annually [5].

The gold standard for the diagnosis of brucellosis is culture isolation [6]. This method is relatively insensitive because *Brucellae* are fastidious organisms; in addition, many patients receive antibiotics before hospital admission, which reduces the chance of obtaining a positive culture. Direct detection of the organism by PCR from clinical samples has been used with varying degrees of success [6]. Thus, the diagnosis of brucellosis often relies on the detection of immunological evidence of exposure to specific antigens using antibody-based blood tests. A variety of commercial kits and protocols exist that are able to measure *Brucella*-specific antibodies. In Georgia, the diagnosis of brucellosis is predominantly based on clinical suspicion and slide (Huddelson) and tube (Wright) agglutination tests, which measure antibodies specific to *Brucella*. Tests that measure other aspects of the immune response to *Brucella* are not routinely used for diagnosis. However, tests based on cell-mediated immune (CMI) responses have been used in the past. For example, a delayed-type hypersensitivity reaction, the Burne skin test, was developed and used for diagnosis of human brucellosis in Georgia until the early 1980s. This test was ultimately abandoned because of the high frequency of hypersensitivity reactions. The diagnosis of *Brucella* using specific skin tests has also been considered as an option elsewhere in the world [7]. Overall, the human immune response to infection with *Brucella* species, particularly the CMI response, is incompletely understood [8]. A number of studies have been conducted on acutely and chronically infected patients that indicate that the immune response to *Brucella* has many facets [9—11]. However, much remains to be elucidated before the intricacies of the human immune response to *Brucella* can be fully understood.

A better understanding of the immune response to this organism could aid in the development of new diagnostic and prognostic methods and could contribute to the development of medical countermeasures, including immunomodulatory therapies or vaccines. Because *Brucella* is an intracellular pathogen, it is likely that a strong CMI response is needed for protection against *Brucella* infection. Indeed, this requirement has been verified in research with mice that are deficient for $CD8^+$ T-cells [12].

For the reasons outlined above, it is crucial to identify patient populations with brucellosis and undertake studies to further characterize the immune response. In the present study, we enrolled 43 patients that had been previously diagnosed with brucellosis and investigated their immune responses using the ELISPOT and ELISA to assess long-term CMI and antibody responses to *Brucella* antigens. We also collected epidemiological and clinical information and laboratory results. The overall aim of the investigation was to establish background information on the immune response to brucellosis in patients in Georgia using existing clinical procedures and diagnostic assays and to revisit the feasibility of conducting CMI response-based diagnosis using new *ex vivo* analogs of the

delayed-type hypersensitivity reaction test (Burne test).

Methods

Patients

Volunteers were recruited, after informed consent had been obtained, from patients who had been treated at the S. Virsaladze Scientific Research Institute of Medical Parasitology and Tropical Medicine (Institute of Parasitology), Tbilisi, Georgia. This hospital has maintained discharge diagnosis records for many years, and the hospital personnel are able to identify patients with a discharge diagnosis of brucellosis. The study involved a single blood draw from adult volunteers (aged >18 years) who had been diagnosed with brucellosis between 3 and 12 months prior to enrollment in the study. The volunteers were asked to assist in completing a Brucellosis Clinical Assessment Questionnaire regarding their clinical signs and symptoms of infection, exposure history, and treatment before and during hospitalization. The patients had received a variety of antibiotic regimens, including streptomycin, tetracycline, chloramphenicol, rifampin, gentamicin or doxycycline. Additionally, patient charts and outpatient logs were reviewed to obtain the clinical and laboratory data.

Laboratory tests at initial diagnosis

Two serological tests, Huddelson's and Wright's tests [1], which measure a specific antibody to *Brucella*, were used to make a presumptive diagnosis of brucellosis. Both tests are antibody agglutination tests. The Huddelson's slide agglutination test provides a qualitative visual record of agglutination, and the Wright's test (tube serum agglutination test, SAT) allows for the measurement of the antibody titer. A titer of 1/200 or higher was considered to be positive.

The full blood count and the erythrocyte sedimentation rate (ESR) data were available for 19 patients at initial diagnosis (Fig. 1). The hemoglobin concentration, total red and white cell counts, lymphocyte count and ESR values that were measured at admission were collected and recorded in the patients' study files upon enrollment.

Sample collection during enrollment into the study

Blood was obtained from all 43 patients that were enrolled in the study and tested for the presence of *Brucella*-specific cellular and humoral immune responses. Venous blood was collected at the volunteer's home into sodium citrate-containing Vacutainer cell preparation tubes (CPTs) and serum tubes (Becton Dickinson™). The blood samples were transported to a laboratory at the National Center for Disease Control and Public Health (NCDC), Tbilisi, and processed within 4 h of collection.

ELISA

The serum for the ELISA was obtained by centrifuging the clotted blood at $1200 \times g$ for 15 min at room temperature. The serum was aspirated into clean tubes and maintained at −80 °C until required.

The antibody responses to *Brucella* LPS were measured using a competitive ELISA (COMPELISA™ kit). The test was provided by the Veterinary Laboratories Agency (VLA), UK, and was performed according to the manufacturer's instructions. Briefly, 20 μl of the test or control sera were placed into each well of a 96-well plate and diluted to 1:6, after which freshly prepared conjugate solution was immediately added. The plate was mixed vigorously for 2 min and incubated for 30 min with constant shaking at room temperature. After extensive washing, each plate was developed using Sigma*Fast*™ OPD tablets (Sigma—Aldrich), and the optical density (OD) was measured (450 nm). The cut-off value was calculated as 60% of the mean OD of four conjugate control wells. Any test sample with an OD equal to or below this value was considered to be positive.

Specific antibody responses to *Brucella*-specific proteins (using Brucellergene™ OCB as an antigen, Synbiotics Corp., Merial, France) were measured using an indirect ELISA developed in-house at the NCDC. Briefly, 96-well plates were coated overnight with brucellergene at a final concentration of 1 μg/ml in PBS and blocked with 5% milk in 1× PBS containing 0.1% Tween-20 (PBST). Serial dilutions (1:50—1:102 400) of each test sample were placed into the preliminary washed wells of a microtiter plate and incubated for 1 h at 37 °C. The wells were washed five times with PBST and incubated for 1 h at 37 °C with an affinity-purified peroxidase-labeled goat anti-human antibody IgG (KPL) at a final dilution of 1:1000 in 5% milk/PBST. The wells were developed using the ABTS peroxidase substrate system and stop solution (KPL). Readings were taken at 405 nm.

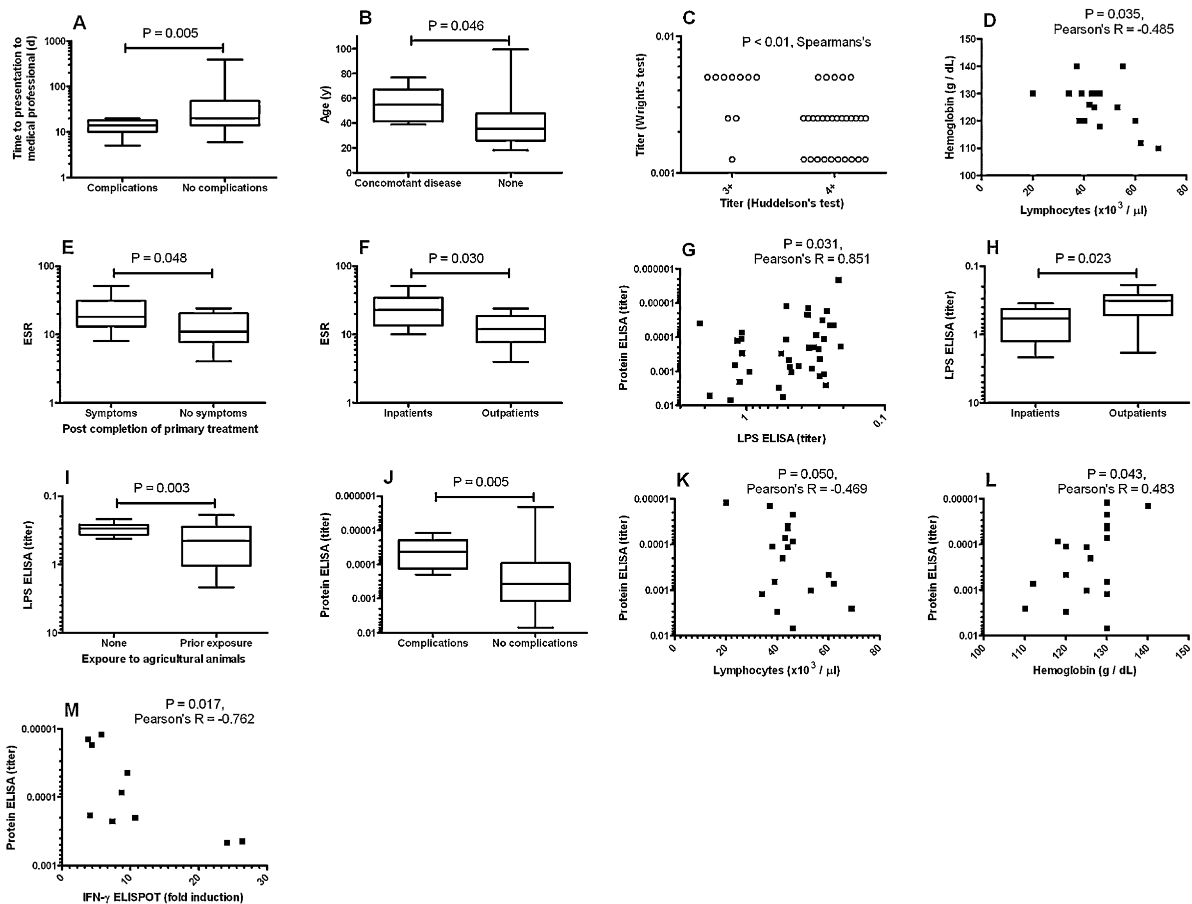

Figure 1 Interactions between the parameters that were measured in patients that were diagnosed with *Brucella* 3—12 months before recruitment to this study. Panel A shows that individuals who reported that they had complications with their disease presented to a medical professional earlier (shown with a box-and-whisker plot and the result from a *T*-test). Panel B shows that older individuals were more likely to suffer concomitant disease (shown with a box-and-whisker plot in which different diseases have been pooled with the results of the ANOVA). Panel C shows the correlation between the SAT tests used to diagnose *Brucella* (shown as a dot plot with the results from Spearman's correlation). Panel D shows a negative correlation between the patients' hemoglobin and lymphocyte concentrations at the time of diagnosis (shown as a scatter plot with the results from Pearson's correlation). Panel E shows that the ESR taken at the time of diagnosis was more likely to be higher in individuals who suffered symptoms after the completion of antibiotic therapy (shown as a box-and-whisker plot with the results from a *T*-test. The antibiotic therapy included a variety of regimens prescribed by medical staff including streptomycin, tetracycline, chloramphenicol, rifampin, gentamicin or doxycycline). Panel F shows that the ERS taken at the time of diagnosis was likely to be higher in patients that were hospitalized (shown as a box-and-whisker plot with the results from a *T*-test). Panel G shows a negative correlation between the patients' antibody titers to *Brucella* LPS and to Brucellergene^{TM} at the time of enrollment (shown as a scatter plot with the results from Pearson's correlation). Panel H shows that the patients' antibody titers to *Brucella* LPS at the time of enrollment was likely to be higher in outpatients (shown as a box-and-whisker plot with the results from a *T*-test). Panel I shows that the patient's antibody titer to *Brucella* LPS at the time of enrollment was likely to be higher in patients who had no prior exposure to animals (shown as a box-and-whisker plot with the results from a *T*-test). Panel J shows that the patient's antibody titer to Brucellergene^{TM} at the time of enrollment was likely to be higher in patients that had complications (shown as a box-and-whisker plot with the results from a *T*-test). Panel K shows a negative correlation between the patients' antibody titers to Brucellergene^{TM} at the time of enrollment and the lymphocyte concentration at time of diagnosis (shown as a scatter plot with the results from the Pearson's correlation). Panel L shows a correlation between patients' antibody titers to Brucellergene^{TM} at the time of enrollment and hemoglobin concentration at the time of diagnosis (shown as a scatter plot with the results of the Pearson's correlation). Panel M shows a negative correlation between the patients' antibody titers to Brucellergene^{TM} and IFN-γ ELISPOT responses to Brucellergene^{TM} at the time of enrollment (shown as a scatter plot with the results from the Pearson's correlation).

ELISPOT assays

Peripheral blood mononuclear cells (PBMCs) were prepared and washed in RPMI medium supplemented with 10% fetal calf serum, penicillin, streptomycin, and L-glutamine. ELISPOTs were performed as described in the manufacturer's instructions (BD™ ELISPOT, Becton Dickinson). Briefly, 0.5×10^6 cells were added to each well of the ELISPOT plates, which were pre-coated with antibodies to interleukin-5 (IL-5) and interferon-γ (IFN-γ). Brucellergene (Brucellergene™ OCB, Synbiotics Corp., Merial, France) was used as a stimulating specific antigen after dialysis against PBS to remove preservative. The antigen was added at 12.5 μg/ml (final concentration), and the plates were incubated overnight at 37 °C in a 5% CO_2, humidified atmosphere. Subsequently, the cells were washed away, and the plates were developed by the addition of an appropriate biotinylated cytokine-detection antibody (to either IL-5 or IFN-γ), which was followed by incubation at room temperature for 2 h. Any unbound antibodies were removed by washing three times with wash buffer, and then streptavidin horseradish peroxidase conjugate was added. After incubation, the enzyme conjugate was removed by washing three times, and the final development of the spots was performed with 3-amino-9-ethylcarbazole (AEC Chromogen kit, Sigma). The spots were enumerated (intensity 3, size 8, gradient 1 and well saturation 83%) using an ELISPOT Reader system ELR04 (Advanced Imaging Devices, GmbH) and the ELISPOT Reader 4.0 software (Autoimmune Diagnostika, GmbH). Concanavalin A (ConA) (2 μg/ml, Sigma Aldrich) was used as a positive control; when cells failed to react to ConA, the samples were not considered for further analysis. Unstimulated (blank) controls were also included for each sample that consisted of cells cultured in medium alone. All of the conditions were assayed in triplicate for each blood sample. The results were considered to be positive when greater than a threefold induction of cytokine excretion was observed over the background. As additional controls, the IFN-γ and IL-5 responses were measured in samples from seven individuals (aged 28—60; six males, one female) with no known prior exposure to *Brucella* and who were not taking any drugs, including antibiotics.

Statistical analysis

The data were analyzed using MINITAB V14.0. For analysis, data were generated for the following parameters: ''Time from symptoms to hospital'', ''Brucellergene OCB ELISA'', and ''CompELISA™''. Furthermore, all of the ELISPOT data were $\log_{10}$ transformed to ensure a better fit to a normal distribution as is required for parametric analysis. Comparisons of two continuous variables were performed using two sample *T*-tests where equal variances were not assumed. Comparisons of more than two continuous variables were performed using a 1-way ANOVA. Explorations of the associations between two continuous variables were performed using Pearson's correlations. The number of ELISPOT responders was compared using Fisher's exact test. The initial diagnostic tests were correlated using Spearman's nonparametric method. The blood test data collected at admission were compared to standard laboratory reference ranges [13,14].

Results

Demographics and clinical characteristics

A total of 43 adult patients (39 males and four females) were enrolled in the study. The initial diagnosis of brucellosis was established based on clinical suspicion and positive slide or tube agglutination tests. Nine patients were hospitalized after presentation; the remaining 34 cases were managed as outpatients.

Occupational exposure to animals was reported by 63% of the patients. Most subjects (84% of the total cohort) had a history of contact with animals before diagnosis, and 37% of the patients had consumed unpasteurized dairy products and undercooked meat in the preceding 2 months. The data obtained from the questionnaire responses suggested that the majority of infections were acquired after direct contact with animals, with 28 (65%) of the 43 patients reporting agriculture-related occupations (shepherd, farmer and butcher) and 16 (37%) reporting the consumption of unpasteurized dairy products before the onset of symptoms. The symptoms included back pain (2 of 26, 7.7%), fever (41 of 43, 95.3%), sweating (33 of 43, 76.7%), malaise (3 of 43, 7.0%), depression (2 of 43, 4.6), general weakness (4 of 43, 9.3%), arthralgia (37 of 43, 86.0%), myalgia (1 of 43, 2.3%), sacroiliitis (2 of 43, 4.6%) and peripheral arthritis (1 of 43, 2.3%).

Seven patients had complications at the time of brucellosis diagnosis, including sacroiliitis (2), peripheral arthritis (2) and epididymo-orchitis (3). Individuals with complications were found to present to the hospital within a shorter time

period after the onset of symptoms. Thus, for the seven patients with complications, the geometric mean time between the development of symptoms and presentation was 12 days, whereas for the 36 patients without complications, the mean interval was 27 days ($p = 0.005$, *T* test for the 7 patients with complications [mean = 1.107 $\log_{10}$ days from onset of symptoms to presentation $\pm$ 0.077 SEM] and for the 36 without complications [mean = 1.447 $\log_{10}$ days $\pm$ 0.075 SEM], Fig. 1A).

The mean interval between time of brucellosis diagnosis and enrollment into the study was 7 months. Twenty-six subjects (61%) were still symptomatic at enrollment. Additionally, documented relapse of symptoms after treatment (*i.e.*, clinical relapse) was recorded in six patients (14%). There was no correlation between the presence of symptoms and the interval between diagnosis and enrollment or the age of the patients ($p > 0.05$). Thirty-one patients did not have concomitant disease (mean age = 38.78 years). The other 12 patients had concomitant diseases; diabetes mellitus ($n = 3$, mean age = 48.26 years) and hypertension ($n = 6$, mean age = 60.28 years) were more common in older people ($p = 0.046$, 1-way ANOVA, Fig. 1B). Additionally, two patients had hepatitis C (one of them complicated by liver cirrhosis) and one had gout.

Laboratory results at the time of primary diagnosis

Slide and Wright SAT test results were available for 39 cases. There was a high degree of concordance between test results ($p < 0.01$, Spearman's correlation, Fig. 1C). Limited analysis had been performed on the blood from the 19 patients (Fig. 1). The hemoglobin concentrations ($n = 19$, mean = 126.1 g/L $\pm$ 1.83 SEM) were negatively correlated with the lymphocyte counts ($n = 19$, mean = 2.546×10^3 cells/μl $\pm$ 0.1941 SEM) at presentation ($p = 0.035$, Pearson's $r = -0.485$, Fig. 1D). The ESR was significantly higher ($p < 0.048$, *T*-test, Fig. 1E) in patients who remained symptomatic after treatment ($n = 11$, mean ESR = 22.9 $\pm$ 4.0 SEM) compared with individuals that were asymptomatic after treatment ($n = 8$, mean ESR = 12.7 $\pm$ 2.5 SEM). The ESR was significantly higher in patients who were admitted to the hospital at presentation ($n = 9$, mean ESR = 25.2 $\pm$ 4.5 SEM) than in those who were not ($n = 10$, mean ESR = 12.7 $\pm$ 2.0 SEM) ($p = 0.030$, *T*-test, Fig. 1F).

Laboratory results at the time of the enrollment into the study

An exhaustive series of statistical comparisons was made between all of the data sets (initial diagnostic parameters, questionnaire data, and enrollment-point laboratory tests). The significant interactions observed in this exhaustive analysis are summarized in Fig. 2. We report all observed positive results but only the negative results that we believe to be important.

Sera from all 43 patients were assessed by ELISA. In total, 35 of 42 (83%) patients were positive for antibodies to *Brucella* LPS by COMPELISA, and 34 of 38 patients (89%) had demonstrable specific antibodies to Brucellergene OCB antigens (Fig. 1G). There was a correlation between the results obtained from the two ELISAs ($p = 0.031$, Pearson's $r = 0.851$). Furthermore, there was evidence for weaker LPS antibody responses (by COMPELISA) in outpatients ($p = 0.023$, *T*-test) and in patients reporting exposure to livestock ($p = 0.002$, *T*-test). There was no association between Brucellergene™ ELISA results and reported exposure to animals or in-patient/out-patient status ($p > 0.05$). However, patients presenting with complications had higher antibody responses to Brucellergene™ than those without complications ($p = 0.005$, *T* test). There was a weak negative correlation between Brucellergene™-specific antibody responses and lymphocyte concentrations at enrollment ($p = 0.050$, Pearson's $r = -0.469$). Furthermore, a significant positive correlation between Brucellergene™-specific antibody responses and hemoglobin concentration was observed ($p = 0.043$, Pearson's $r = 0.483$) (Fig. 1K and L). However, no significant correlations between hemoglobin, lymphocyte concentrations and COMPELISA results ($p > 0.05$) were demonstrated.

Blood samples from 15 patients (35%) were assessed for CMI responses to brucellergene OCB. All 15 patients were positive for antigen-specific elicitation of IFN-γ (100%). Only 14 valid patient ELISPOTS were obtained for IL-5, of which 8 (57%) were considered to be positive. None of the seven control subjects (those with no history of *Brucella*) elicited measurable cytokine responses to *Brucella*-specific antigens. There were significant differences between the antigen-specific cytokine responses for patients and unexposed controls (IL-5, $p = 0.018$; IFN-γ, $p < 0.001$, Fisher's exact test.) There was a significant inverse correlation between brucellergene-specific antibody and IFN-γ ELISPOT responses ($p = 0.017$, Pearson's $r = -0.762$) (Fig. 1) but no correlation with IL-5 ELISPOT responses ($p > 0.05$). Neither cytokine response

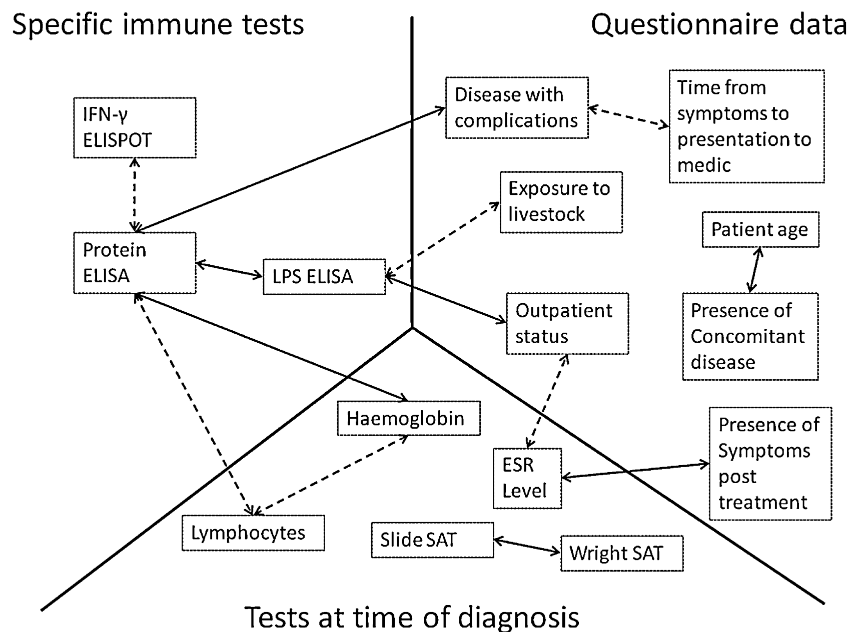

Figure 2 A diagrammatic representation of the significant interactions between the characteristics of *Brucella* patients in Georgia. These characteristics have been subdivided into the following categories: questionnaire data (whether the disease was complicated, the period of time elapsed from onset of symptoms to presentation to health professionals, whether patients had exposure to livestock before infection, patient age at the time of diagnosis, presence of concomitant disease, whether the patient was treated as an outpatient and whether they suffered disease symptoms post treatment), the results of clinical tests administered at the time of diagnosis (Slide or Wright SAT, ESR, hemoglobin and lymphocyte counts) and specific immunological measurements obtained 3—12 month post diagnosis (antibody ELISA to Brucellergene™ or LPS and IFN-γ ELISPOT responses to Brucellergene™). The interactions between these parameters are shown as double-ended arrows, and negative interactions are represented by broken double-ended lines. The full details of these interactions and the statistical analysis are shown in Fig. 1.

was correlated with the COMPELISA responses ($p > 0.05$).

There were no correlations between any aspects of humoral or CMI response and the time between the onset of symptoms and enrollment ($p > 0.05$ in all cases). Furthermore, we observed no differences in antibody or CMI response when comparing those that were symptomatic at the time of enrollment and those who were not ($p > 0.05$ in all cases).

Discussion

In this study, we explored long-term immunity in a cohort of individuals that had been previously diagnosed with brucellosis, many of whom were still symptomatic. Although confirmation of the diagnosis by positive culture was not available for any of the subjects, our data support the diagnosis of brucellosis in the majority of cases.

The patients reported a variety of symptoms, and there was no evidence of a correlation between different symptoms and the clinical and diagnostic parameters that were measured. These findings are consistent with published data on brucellosis as a complex disease with a multifaceted clinical presentation [3,15]. Additionally, we found several results that could easily be explained. Patients with complications were found to present to the hospital within a shorter time period after the onset of symptoms; such individuals may already have been in contact with medical professionals or had more severe disease. Individuals with concomitant disease were more likely to be older; the disease burden was greater with age. ESR values were greater in individuals who were symptomatic after treatment and in in-patients, which are both factors that may indicate more severe disease. In previous studies, ESR has been shown to be linked with cytokine production [16]; however, in the previous work, the cytokine levels were measured during the progression of the disease, whereas our measurements were taken post-infection. For these reasons, we believe that these data sets support each other. Other results, such as the relationship between lymphocyte concentration, hemoglobin concentration and the humoral response to brucellergene, have no obvious explanation. Analysis

of questionnaire and clinical data generated at the time of initial presentation increases our confidence that individuals had been infected by *Brucella*, and the symptoms in Georgia are congruent with our current understanding of the disease [2]. Unfortunately, it was not possible to accurately determine the period of time that elapsed between initial diagnosis and relapse in this study. Previous data have shown that weak immune responses to *Brucella* can affect the likelihood of relapse [17].

At enrollment into the study, a large proportion (>80%) of patients were serologically positive for *Brucella* LPS and protein (Brucellergene^{TM}), and *Brucella* protein was detected by the CMI assay. This observation provides us with further confidence that our cohort of patients was originally infected with *Brucella*. Positivity in the CompELISA assay was determined as an OD value of greater than 60% of the positive control sera, according to the manufacturer's recommendations. However, this assay has not been validated for the diagnosis of human brucellosis, and a full assessment of the utility of the CompELISA for use in human diagnosis was not within the scope of this study. Nevertheless, the existence of strong CompELISA (LPS-specific antibody) and Brucellergene^{TM} (cytosolic antigen-specific antibody) responses suggests that a sustained and robust memory response and/or continued exposure to antigen exist. Furthermore, we observed a good correlation between the results of the ELISA for antibody response to LPS and to Brucellergene^{TM}; the majority of patients mounted specific antibody responses to both antigens. This observation indicates that after presentation and treatment, the humoral immune system is capable of recognizing a variety of *Brucella* antigens. Furthermore, it indicates that the humoral immune response to *Brucella* antigens is long-lived, as these responses were detected up to 12 months after initial diagnosis and treatment of brucellosis. The longevity of anti-*Brucella* responses is well documented, and persistent SAT titers of greater than 1:320 have been recorded in patients with resolved infection [18]. Previous studies have investigated the diagnostic/prognostic potential of different antibody-based techniques for human brucellosis. Baldi et al. [19] observed that ELISA results correlated well with clinical outcomes (resolution, persistence and relapse of signs and symptoms). In contrast to the study described by Baldi et al., we did not observe a significant positive correlation between the immune response and the relapse of symptoms, although this lack is likely a reflection of the low power of our study. In addition, this study concluded that, although LPS would be an excellent candidate for diagnostics, due to the cross-reactivity of the LPS protein, LPS-depleted cytosolic extract might be a preferable method. With these observations in mind, it appears to be more likely that many of our patients had not cleared the infection completely. Mantecon et al. [20] explored antibody subtypes in detail and found similar results. Memish et al. [21] found that both IgG and IgM should be measured to assure good specificity and sensitivity. Our simple ELISAs for LPS and protein are not isotype-specific.

In our studies, an association between an antibody response to *Brucella* LPS (but not Brucellergene^{TM}) and outpatient status (outpatients have a greater response) and patients reporting contact with livestock was observed (those with contact had a greater response). We also observed an association between the Brucellergene^{TM}-specific responses and patients reporting complications (in which those with complications had a greater antibody response). The weaker antibody responses to *Brucella* LPS in patients that reported contact with livestock are difficult to explain; however, this finding might be due to an immune tolerance to a previously recognized antigen. Stronger brucellergene-specific antibody responses in patients that reported complications may reflect a more protracted course of disease and longer-term exposure to *Brucella*, which allowed for recognition of a more diverse range of antigens. Interrogation of the initial clinical laboratory tests and humoral immune responses upon enrollment in the study revealed a weak inverse correlation between total lymphocyte counts and antibody responses to Brucellergene^{TM}. This association could reflect the maturity of the immune response and be related to the changing nature of antigen recognition as infection progresses from initial antigen encounter to a more chronic disease. Overall, these observations regarding humoral immunity suggest that a brucellergene/LPS differential diagnostic for human brucellosis could potentially be used in future studies to improve the understanding of human disease progression.

Measurements of CMI responses, such as intradermal delayed-type hypersensitivity reactions (allergic skin test), have been investigated for use as diagnostic methods for animal brucellosis [22,23], and analogous assays have been developed that measure the *ex vivo* elicitation of IFN-γ in response to stimulation with skin test antigens [24]. For this study, ELISPOT assays that measure the production of IFN-γ and IL-5 by *ex vivo* stimulated PBMCs were used to investigate the CMI responses

of patients. All of the patients tested were capable of eliciting an IFN-γ response (Th1), but only 57% produced a measurable IL-5 response (Th2) upon recall stimulation. Control subjects revealed no increase in the production of either IFN-γ or IL-5 in response to specific *Brucella* antigen stimulation, which suggests that the assays were measuring responses that are specific to brucellosis infection and could provide a basis for a valuable diagnostic approach. However, it should be noted that the sample size in these studies was small and further investigations would be necessary to determine the diagnostic and analytical sensitivity and specificity of these approaches before they could be used for clinical diagnosis of brucellosis. The production of IFN-γ from all (100%) patients and IL-5 from only 57% of patients is consistent with the expected Th1 bias that is associated with *Brucella* infection [25]. In addition, we observed an inverse correlation between IFN-γ production in response to brucellergene stimulation and demonstration of brucellergene-specific antibodies. This effect may be related to Th1/2 switching in these patients, which suggests that the antibody responses are strongest where the cellular responses are relatively weak. These data may relate to different stages of immune development or disease progression and resolution in these patients. A shift from a dominant Th1—Th2 response has previously been correlated with the development of chronic infection [26]. Furthermore, targeted investigations are necessary to fully elucidate these findings where Th1/2 biasing would be measured during the acute stage of the disease.

These data also raise the possibility that components of Brucellergene (or alternative *Brucella* antigens) may warrant further investigation as diagnostic antigens for human brucellosis and, particularly, as stimulating antigens for CMI assays. Brucellergene contains two known immunogens (*Brucella* bacterioferritin and P39, a putative periplasmic substrate-binding protein [27]) [28]. Brucellergene (sometimes termed brucellin) has been considered favorably for the development of diagnostics in the past. Cellular responses have been investigated in confirmatory diagnostic testing in the field of animal health and disease control in which Brucellergene was used in an intradermal delayed-hypersensitivity test to indicate prior exposure and adaptive immunity to cytosolic antigens of *Brucella* [29,30]. *In vitro* tests to measure specific IFN-γ from the supernatants of stimulated cells were also assessed for this purpose. The rationale for the assessment of cellular responses was to compare cellular responses to antibody responses to *Brucella* proteins or to *Brucella* LPS, thus possibly providing a more confident prediction of active infection. However, varying degrees of success were observed with this approach. The traditional serodiagnosis of *Brucella* relies on the detection of antibodies to the immuno-dominant LPS. Assessment of the cellular and serological response to proteins would offer an alternative specificity of antigens for diagnosis. This study sought to investigate the utility of protein antigens for this purpose. Weynants et al. [23] found the test to be useful as a confirmatory diagnostic test when a false positive serological reaction was observed, whereas Kittelberger et al. [22] concluded that the test had sub-optimal specificity. Giambartolomei et al. [11] found that IFN-γ levels were dependent on whether the disease was chronic or acute and that these responses decayed over a period of greater than a year. In our study, all of the patients were enrolled less than a year after the initial diagnosis; therefore, we were not able to confirm whether these responses diminished over protracted periods of time. Although we did observe variation in IFN-γ responses, we did not observe an association between these responses and clinical manifestation of the disease. It is highly likely that this lack was reflective of the small sample size that was used in the ELISPOT analysis, and we would suggest that our data neither confirm nor deny the observations of Giambartolomei et al. Furthermore, our study lacked longitudinal sampling, which would have allowed for more power when investigating whether an individual's response declined over time. Ultimately, these tests have not provided the broad utility and simplicity that is typical of serum-based diagnostics, and their use in animal disease control strategies remains relatively restricted to confirmatory diagnostic testing and research investigations rather than high-throughput screening. An analogous cellular response test for the diagnosis of brucellosis in humans, the *Brucella* allergic skin test, was previously used in Georgia. As scientific methods and our understanding of the immune system have improved since the *Brucella* allergic skin test was last used, further pursuit of these concepts for diagnostics could be beneficial. Currently, specific validated diagnostic tests for human brucellosis that rely on measurement of cellular immune effectors are not available. Our findings indicate that a strong adaptive immune response is measurable in the majority of patients and that further interrogation of this response may reveal data that are pertinent to treatment outcome or prognosis. Moreover, such tests may be useful for predicting the outcome of disease and/or treatment regimens [31].

Conclusions

This small study demonstrates persistent humoral and cellular immune responses in patients with brucellosis and indicates a need for further investigation. Furthermore, it raises the possibility of using responses to *Brucella* protein antigens (cellular and humoral) as diagnostic targets. In particular, the measurement of antibody and IFN-γ responses to Brucellergene^{TM} protein epitopes may be worthy of further investigation as an alternative or adjunct to current diagnostics. Potential *Brucella* vaccine candidates could also be assessed in these assays.

Funding

No funding sources.

Competing interest

We have no conflict of interest to declare.

Ethical approval

Not required.

Disclaimer

The opinions, interpretations, conclusions, and recommendations are those of the authors and are not necessarily endorsed by the U.S. Army.

Acknowledgements

The clinical study protocol received ethical approval from the U.S. Army Medical Research Institute of Infectious Diseases (USAMRIID) and the NCDC Institutional Review Boards (IRBs). Samples from healthy volunteers were collected under a protocol approved by the Defense Science & Technology Laboratory CBD Independent Ethics Committee. All of the subjects provided informed consent to participate in the study before enrollment. We thank Mr. David Elliott, Dr. Tatia Kuchuloria, Ms. Amanda Lane and Mr. Glyn Choules for their assistance with this study. This work was funded by the British Ministry of Defense and the Joint Science and Technology Program in Chemical and Biological Defense, Defense Threat Reduction Agency, USA.

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