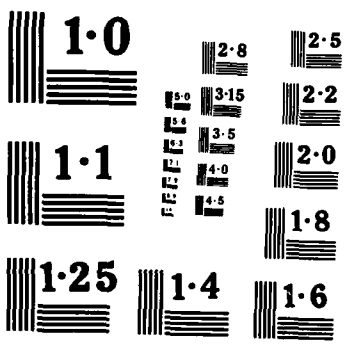
AD-A158 095 STUDIES ON THE ANTIGENIC COMPOSITION OF COXIELLA BURNETTI(U) WASHINGTON STATE UNIV PULLMAN D J HINRICHS 01 DEC 81 DADA17-73-C-3090 1/1

UNCLASSIFIED F/G 6/5 NL



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FINAL REPORT

DAVID J. HINRICHS

December 1981

STUDIES ON THE ANTIGENIC COMPOSITION OF COXIELLA BURNETII

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND

Fort Detrick, Frederick, Maryland 21701

Contract #DADA17-73-C-3090

Washington State University
Pullman, Washington 99164

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REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER	2. GOVT ACCESSION NO. AD-A158095 / 3. RECIPIENT'S CATALOG NUMBER
4. TITLE (and Subtitle) Studies on the antigenic composition of Coxiella burnetii	5. TYPE OF REPORT & PERIOD COVERED FINAL REPORT 6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) David J. Hinrichs, Ph.D.	8. CONTRACT OR GRANT NUMBER(s) DADA17-73-C-3090
9. PERFORMING ORGANIZATION NAME AND ADDRESS Washington State University Pullman, Washington 99164	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS 62776A.3M162776A841.00.083
11. CONTROLLING OFFICE NAME AND ADDRESS U.S. Army Medical Research & Development Command Fort Detrick, Frederick, MD 21701	12. REPORT DATE December 1, 1981 13. NUMBER OF PAGES 10
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)	15. SECURITY CLASS. (of this report) Unclassified 15a. DECLASSIFICATION/DOWNGRADING SCHEDULE N/A

16. DISTRIBUTION STATEMENT (of this Report)

Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Coxiella burnetii; Q-fever; antigenic analysis; chemical composition; cell-mediated immunity; animal model; in vitro analysis.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This final report recounts the experimental findings that support the postulate that Coxiella burnetii infections are controlled by nonhumoral cell mediated immune mechanisms.

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Background

Contract DADA-17-73-C-3090 entitled "Studies on the Antigenic Composition of "Coxiella burnetii" was initiated due to the collaborative interests of Drs. David J. Hinrichs, Louis P. Mallavia and John P. Heggers. Dr. Mallavia had worked extensively on the basic physiology of Coxiella burnetii in David Paretsky's lab during his Ph.D. training and continued these studies at Washington State University. At the initiation of the contract John Heggers was a graduate student (active Army) interested in infectious disease and the immune system. The lack of any published investigations on Q-fever that related specifically to non-antibody mediated immunologic events was considered to point to an area in need of investigation given the obligate intracellular parasitism of C. burnetii. This led to our first series of studies that dealt with the development of cellular immunity in the guinea pig following infection or following vaccination with formalized, whole cell, vaccines. Our in vitro measurements of cell-mediated immunity were the first to establish that nonhumoral immunity may be of importance in the immunity that develops to C. burnetii. From this point in time we embarked on a series of studies designed to evaluate the contribution of cellular immunity in C. burnetii infections. In addition it was also deemed important to evaluate vaccine effectiveness of preparations of C. burnetii whole cells and of subcellular elements. The body of this final report summarizes our findings in these areas and fully establishes the role of cell-mediated immunity in the control of rickettsial organism C. burnetii.

Existence of a Specific Cell-Mediated Immune Response in Guinea Pigs

The guinea pig response to C. burnetii as determined by migration inhibition assay clearly demonstrated the development of cell-mediated immunity in vaccinated guinea pigs as well as in guinea pigs recovered from infection caused by C. burnetii Phase I, nine mile strain. Guinea pigs vaccinated with 0.1 to 1.0 mg of a formalin-inactivated whole cell preparation (WCP) of C. burnetii developed positive skin test reactivity and the skin test response was in direct correlation with the macrophage migration inhibition assay. The antigenic specificity was also found in Trichloroacetic acid (TCA) extracts of Phase I C. burnetii. The migration inhibition response of vaccinated animals was readily detectable two weeks following immunization and continued to be evident through the three month evaluation period. Guinea pigs infected with Phase I C. burnetii, developed a cell-mediated immune response that could also be measured by migration inhibition assay. The antigens employed for this detection were WCP and the TCA extract. Of the two antigenic preparations the TCA extract allowed a more reproducible assay of cell-mediated immunity in vitro than did the formalin inactivated WCP. Some of these initial findings in the guinea pig were published in 1974.

Existence of Cell Mediated Immune Response in Humans

It became apparent during our studies on C. burnetii that many of our colleagues adhered to the concept of protective humoral (not cellular) immunity in Q-fever. Consequently vaccine development when "successful" would be technically evaluated simply by serologic conversion. The study of the immune response to C. burnetii in humans had been limited to skin test reactions and the serologic evaluations following vaccination or infection. In collaboration with graduate student Thomas Jerrells we evaluated the cellular immune reponse in a number of individuals that had worked with C. burnetii in the laboratory,

had a previous history of Q-fever with positive serologic confirmation or had no experience with C. burnetii. We were also able to evaluate the development of cell-mediated immunity to C. burnetii during the acute and convalescent phase of Q-fever that occurred in a member of Dr. Mallavia's research team. The assay for cell-mediated immunity chosen for the human study was the lymphocyte transformation test. We felt that this test would have greater applicability due to technician independent measurements than those associated with macrophage migration assays. Furthermore the amount of blood needed for lymphocyte transformation assays is one-tenth of that needed for leucocyte migration assays.

In these studies cellular immunity was demonstrated in human subjects with various past histories of exposure to the organism by using lymphocyte transformation assays. Individuals with histories indicating exposure to C. burnetii up to eight years before the study demonstrated marked lymphocyte transformation in vitro to whole-cell antigens consisting of formalin-killed C. burnetii phase I and phase II. These individuals also demonstrated a marked lymphocyte response to the trichloracetic acid-soluble phase I antigen. The individual who acquired Q-fever during the study and one individual who received an experimental Q-fever vaccine 4 years earlier were also evaluated by the lymphocyte transformation assay and were found to have positive responses. The one individual we examined during the acute and convalescent phase of Q-fever in some cases had a lymphocyte transformation response 4s marked as that induced by the T cell lectin Phytohemoglutinin. It was also found that phase I trichloracetic acid-soluble material was capable of acting as an antigen in the assay, whereas phase II trichloracetic acid-soluble material did not contain any antigenic material capable of causing lymphocyte transformation. The complete phase I trichloracetic acid-soluble antigen, which was found to consist of protein and carbohydrate, was chemically fractionated into monospecific fractions. The fraction treated to eliminate carbohydrate was the only fraction found to elicit an in vitro response.

Many of the individuals that had a positive lymphocyte transformation response to C. burnetii antigen had no detectable antibody titers. We evaluated our initial studies in the guinea pig and the data obtained from the human studies and postulated that cell-mediated immunity may play the deciding role during a C. burnetii infection. Our next phase of work was designed to establish specifically the role that cell-mediated immunity may play in combating Q-fever infections.

Mechanism of Immunity to C. burnetii as Determined by In Vitro Analysis

In order to evaluate the role of nonhumoral immunity in C. burnetii infection we initially designed experiments to answer this question in vitro. The system employed the guinea pig macrophage as the target cell. Replication of C. burnetii within guinea pig macrophages could be readily detected by microscopic evaluation of stained macrophages monolayers. Additionally we employed an assay of specific immunoprecipitation of C. burnetii following growth in macrophage cultures to which we added ^{3}H-thymidine. This increased radioactivity in the immunoprecipitate directly correlated with an increase in C. burnetii numbers determined microscopically or by egg LD_{50} assay.

In this series of studies we found that C. burnetii is readily phagocytized by macrophages maintained in vitro. This phagocytosis does not lead to destruction of the organism but rather to intracellular multiplication within the

macrophage. Specific antiserum added to the macrophage culture before or after infection, or reacted directly with C. burnetii organisms, fails to control subsequent intracellular replication. Macrophage cultures can be treated with lymphocyte products so that intracellular multiplication by C. burnetii is resisted. These lymphocyte products can be obtained from the culture supernatants of sensitized lymphocytes and antigens or from culture supernatants of Concanavalin A and normal lymphocytes. The activation activity of the lymphocyte supernatants paralleled their content of migration inhibitory factor.

The results of these in vitro studies did not rule out a role for antibody in controlling C. burnetii infections. The studies did however demonstrate conclusively that activated macrophages were a required component of the successful anti-C. burnetii immune system. These observations allowed us to postulate that lymphocyte derived macrophage activating factors were involved in immunity to C. burnetii and that measurements of cellular immunity would be better indications of immune status than would humoral assays.

If immunity to C. burnetii depended on specific T cell recognition of antigen with release of lymphokines and eventual macrophage activation then successful vaccine development would depend on the activation of this non-antibody arm of the immune response. Because of the argument surrounding this issue and in order to more rigorously establish the point we felt that in vivo studies had to be conducted. For these final series of experiments on immunity to C. burnetii we evaluated and then employed the mouse as an experimental animal.

Response of Normal Mice to Infection with C. burnetii

We found that the response in mice to infection with C. burnetii could be followed by qualitative evaluation of C. burnetii from stained spleen impression smears or more quantitatively by direct particle count on homogenized tissue. Following infection with 10^5 C. burnetii particles we found that rickettsial growth is virtually unrestricted during the first four days post infection when levels reached 10^9 rickettsiae per 100 mg splenic tissue. By day 4, large numbers of extracellular rickettsiae were readily apparent in stained spleen preparations. At days 5-7, the amount of extracellular rickettsiae had diminished and the apparent number of intracellular rickettsiae increased, and by day 10, essentially all the rickettsiae were intracellular. Clearance of the rickettsiae appeared to occur during the second week of infection. Agglutinating antibodies were demonstrable within the first week after infection, and the titer continued to rise throughout the three weeks of observation.

Response of Infected, Recovered Mice to Reinfection

If the clearance of C. burnetii is immunologically mediated then these recovered animals should show a secondary immune response when subsequently challenged with C. burnetii. When these rechallenged experiments were performed it became very obvious that a state of immunity could develop in mice and that the state of immunity apparently caused rapid clearance of C. burnetii in rechallenged animals. In order to establish the mechanism of immunity to C. burnetii in mice we chose two experimental approaches. In the first case we attempted to alter the developing immune response with immunosupressive compounds, and in the second case we evaluated antibody and lymphoid populations, obtained from C. burnetii immune mice in standard passive transfer assays. We employed normal and nude mice as recipients of these immunologic reagents.

The experiments which measured the effects of immunosupressive reagents on the course of C. burnetii infection showed that anti-lymphocyte serum, anti-macrophage serum or the intraperitoneal administration of silica substantially altered the clearance of C. burnetii from the spleen or liver of treated animals. Of greater interest was the observation that cyclophosphamide treatment prior to infection was found to cause 100% mortality in C. burnetii infected mice within ten days post infection. When silica was administered intravenously it was found that a lethal event could be induced, but the percent mortality was much lower than that achieved with cyclophosphamide. Treatment with the various immunosuppressive agents prior to infection with C. burnetii was found not to alter antibody production to a significant degree when compared to infected control animals. This last observation could be correlated with our findings that C. burnetii possesses a potent B lymphocyte mitogen and consequently a T cell independent antibody response could be expected to develop in vivo. Thus titers of antibody could developed in immunosupressed animals at a time when infection due to C. burnetii was continuing unchecked.

Effects of Antibody in C. burnetii Infection as Determined by Passive Transfer

The role of antibody in infectious disease is tested, in many experiments, by mixing specific antibody with the infectious unit in vitro and then determining infectivity by in vivo challenge with the mixture. Alternatively, the role of antibody on the infectious disease process is determined by in vivo passive transfer experiments in which specific antibody is given to a suitable experimental animal. The antibody recipient is subsequently challenged with the viable organism, and if the disease develops, the role of antibody in the clinical disease is assumed. There are potential problems which can develop from this type of experimental design when one studies diseases caused by intracellular parasites. In the normal clinical course of these diseases the microorganism gains entrance and develops intracellularly before the development of an immune response. Since antibody is poorly permeable to living cells, an experimental design which includes in vitro mixing of parasites with specific antibody or in vivo challenge after transfer of antibody may lead to erroneous assumptions as to the role of antibody in infectious diseases caused by the intracellular parasites.

To test a role for antibody in C. burnetii infections, we prepared a pool of specific antibody from infected, recovered mice. This antiserum was administered intravenously to normal mice before, simultaneously with, or after C. burnetii challenge. Immune serum, administered to normal mice 24 h before challenge with C. burnetii, appeared to accelerate the development of resistance. An increased clearance rate could be measured in these serum recipients 1 week postinfection. Simultaneous administration of immune serum and C. burnetii did not affect the normal clearance rate of rickettsiae from the spleens of infected mice during week 1, but did enhance clearance of the organism by 14 days postchallenge. Passive transfer of immune serum 24 h after challenge of normal mice with viable C. burnetii had no effect on rickettsial growth within the spleens of animals treated in this fashion. Treatment of athymic mice with immune serum 24 h before challenge with C. burnetii had no effect on rickettsial multiplication within the spleens of these T-cell-deficient animals.

When one compares the effect of passively administered antibody on C. burnetii infections in normal and athymic mice the role for humoral immunity can be evaluated. No direct role for antibody in combating C. burnetii can be supported. From the results of experiments with normal and athymic mice we feel that the only possible role for antibody could be in situations were levels of

specific antibody exist before infection. In these situations antibody may combine with C. burnetii and accelerate the development of cell-mediated immunity. This may be accomplished by more efficient processing of C. burnetii-antibody complexes than with C. burnetii itself. In the primary experience (in the absence of antibody) the elimination of C. burnetii would be dependent on the development of activated macrophage via lymphokine released from antigen sensitive T lymphocytes.

In the final series of experiments on immunity to C. burnetii we evaluated the role of lymphocytes directly by passive transfer of immune cells and by reconstitution of athymic mice with thymic tissue. In these studies spleen cells were obtained from C. burnetii infected-recovered animals or from mice immunized with phase I C. burnetii formalized vaccines. The spleen cells were transferred into normal or athymic recipients and these cell recipients were then challenged with viable C. burnetii. Clearance rates were determined at 7 and 14 days and it was readily determined that recipients of immune spleen cells had cleared all signs of challenge by 14 days. Most recipients had very few rickettsial bodies at day 7. Egg challenge indicated that these rickettsia were not viable. Treatment of the spleen cells before transfer with anti-thymocyte serum eliminated any transferred immunity. These experiments were consistent with a crucial T cell role in the development of immunity to C. burnetii. We expanded on this latter point in a study on the effects of C. burnetii in athymic mice. Athymic (nu/nu) mice can not control a challenge dose of C. burnetii and the majority of these infected mice die. However if athymic mice are employed as recipients of immune spleens cells rapid clearance of the C. burnetii challenge is seen. If nude mice are given C. burnetii specific antibody from infected recovered mice and then challenged with C. burnetii overwhelming C. burnetii infection is seen in all test animals. Finally, when nu/nu mice are reconstituted with thymocytes prior to C. burnetii challenge they develop a state of immunity that clears the challenge at a rate equivalent to that seen in normal (+/nu) littermates.

Summary Statement

Our entire approach throughout these studies has dealt with the question of the immunologic mechanism of resistance in Q-fever. We feel that our studies conclusively show that immunity to C. burnetii depends on the development of activated macrophages which in turn only develop when T cell produced lymphokines are released. Without the T cell component no immunity can develop. All of our experiments are consistent with this interpretation and we are not aware of any published work on C. burnetii immunity in vivo that is at variance with this hypothesis.

The need for T cell involvement in Q-fever warrants an evaluation of any putative sub-unit vaccine (or more contemporarily) a cloned "protective antigen" in light of humoral vs. cellular regulation systems. Most "pure antigens" do not elicit the development of nonhumoral cellular immunity without the use of complex adjuvants. Hypothetically it is not in keeping with an efficient immune system to respond to innocuous antigen by developing populations of activated macrophages. Thus subunit vaccines do not have a high prediction for success (in diseases caused by C. burnetii type infectious mechanisms) unless the immunologic control point of cellular vs. humoral responsiveness can be elucidated. Our concern over this point has kept our studies of C. burnetii and immune mechanisms on direct as well as more circuitious approaches to the solution of this problem. We are continuing our studies in this regard and

feel that an understanding of the initial phase of antigen (pathogen) interaction with the cells of the immune system will be required in order to eventually control and promote the induction and expression of cell-mediated immunity.

Graduate Students Receiving Training and Support

Throughout the contract period, graduate student research has been an important component of our success. These graduate students received "hands on" experience with a host-parasite system that is technically demanding and requires ability and patience. This experience has served them well and each of the graduate students supported by the contract are continuing as professional scientists and have continued their research on problems of infectious disease and regulation of the immune response. Many of these individuals assisted with the C. burnetii project while also maintaining research projects that were concerned with control mechanisms that could be eventually applied to our investigations of C. burnetii.

These individuals are listed below with the date of their degrees and current professional position. Finally a list of published works by these individuals is included:

John Heggars	Ph.D.	(1972)	Professor of Surgery, University of Chicago, Chicago, IL.
Thomas R. Jerrells	Ph.D.	(1976)	Research Immunologist, Walter Reed Army Medical Hospital, Washington, D.C.
Howard V. Raff	Ph.D.	(1977)	Assistant Professor, Department of Microbiology and Immunology, University of Washington, Seattle, WA.
Robert J. Kearns	Ph.D.	(1978)	Research Immunologist, The Samuel Roberts Noble Foundation Inc., Ardmore, OK.
Robert C. Humphres	Ph.D.	(1979)	Research Associate, Stanford Research Institute, Menlo Park, CA.

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