INFECTION AND IMMUNITY, Feb. 1975, p. 280-286

Vol. 11, No. 2
Printed in U.S.A.

Detection of Long-Term Cellular Immunity to *Coxiella burneti* as Assayed by Lymphocyte Transformation

T. R. JERRELLS,* L. P. MALLAVIA, AND DAVID J. HINRICHS

Bacteriology and Public Health, Washington State University, Pullman, Washington 99163

Received for publication 26 September 1974

Delayed hypersensitivity to the antigens of *Coxiella burneti*, Nine Mile strain, 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. burneti* up to 8 years before the study demonstrated marked lymphocyte transformation in vitro to whole-cell antigens consisting of formalin-killed *C. burneti* phase I and phase II. These individuals also demonstrated a marked lymphocyte response to the trichloracetic acid-soluble phase I antigen. One 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. It was also found that phase I trichloroacetic acid-soluble material was capable of acting as an antigen in the assay, whereas the phase II trichloroacetic acid-soluble material did not contain any antigenic material capable of causing lymphocyte transformation. The complete phase I trichloroacetic 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.

The mechanism(s) involved in acquired immunity associated with intracellular rickettsial parasitism remains ill defined. Antibody of the agglutinating and of the complement-fixing type is produced after infection with *Coxiella burneti* as well as with other rickettsiae (8). These antibodies, associated with Q fever infections, have been well studied in humans and experimental animals (2, 8, 14, 18). In addition, serological assay has demonstrated that *C. burneti*, unlike other rickettsiae, exhibits a unique, host-controlled, antigenic phase variation (19). Organisms isolated from natural infections of man and animals are found to be in phase I. Passage of these organisms in embryonated eggs results in the loss of the capsular-like phase I antigen (6, 7). This host-controlled change in the antigenic character results in the serological demonstration of cell wall-associated phase II antigen(s) (8). These observations would support the contention that the phase I antigen promotes the intracellular parasitism observed with *C. burneti* in man and experimental animals.

Antibodies are readily detected after vaccination (14, 15) or infection (11) with *C. burneti*. The fact that these antibodies are transient (8, 14) and are first directed against phase II antigens and only later against the phase I antigen suggests that protection against reinfection may not coincide with the humoral response. It is apparent from skin test studies in humans (13, 15) and from recent work in guinea pigs (11) that development of delayed hypersensitivity occurs after infection with *C. burneti*. The lymphocyte-mediated hypersensitivity that occurs in Q fever implies that long-lived immunity may be a cellular immune event in this disease.

The purpose of this study was to determine the existence and continued levels of cell-mediated immunity in individuals with various histories of exposure to *C. burneti*. The levels of cell-mediated immunity were evaluated by a lymphocyte transformation assay. Lymphocyte transformation was also used to evaluate the contribution of isolated rickettsial phase antigens to the observed lymphocyte-mediated response.

(This paper was submitted by T.R.J. to the Graduate School of Washington State University in partial fulfillment of the requirements for the M.Sc. degree.)

MATERIALS AND METHODS

Cultivation and purification of the organism. *C. burneti*, Nine Mile strain, phase I and phase II, were cultivated in the yolk sac of 6-day-old embryonated

hen eggs as previously described (11). Upon death of approximately 50% of the inoculated embryos, the yolk sacs were aseptically harvested, and the organisms were purified from the yolk material by the method described by Ormsbee (16). To insure purity of each phase type, density gradient sedimentation studies were performed on aliquots of purified samples by the procedure of Hoyer et al. (12), with continuous cesium chloride gradients or 35 to 75% discontinuous sucrose gradients prepared in water.

Antigen preparation. Antigens used throughout this study consisted of whole-cell preparations of phase I and phase II organisms, trichloroacetic acid-soluble material obtained from both phase I and phase II organisms, and fractions of the trichloroacetic acid-soluble material obtained from phase I organisms.

Whole-cell antigens were prepared by taking aliquots of the purified rickettsiae and treating them with 0.15 M NaCl containing 0.6% formalin. After incubation at room temperature for 24 h, the formalin-treated organisms were washed three times in sterile 0.15 M NaCl and finally resuspended in a sufficient volume of 0.15 M NaCl to achieve the desired concentration. Whole-cell preparations of *C. burneti* phase I and phase II (WCP-I and WCP-II) were expressed in terms of micrograms (dry weight) per milliliter, which was estimated by using a Klett-Summerson colorimeter after standardization.

The soluble phase I and phase II materials were obtained by the method of Anacker et al. (2). Briefly, the procedure involved suspending phase I or phase II organisms in 0.15 M NaCl to a concentration of approximately 1 mg (dry weight) per ml. To this suspension an equal volume of cold 0.5 N trichloroacetic acid was added, and the mixture was stirred with a magnetic stirrer for 3 h at 4 C. This suspension was then centrifuged for 15 min at 6,100 $\times$ *g*, and the supernatant was recovered. This material was dialyzed for 24 h against running tap water and 24 h against several changes of distilled water. After dialysis, the fractions were concentrated with an Amicon filter UM20E designed to retain substances of molecular weight greater than 20,000. This material, designated TCA-I or TCA-II, was adjusted to a standardized concentration based on protein concentration and stored at -70 C.

The trichloroacetic acid-soluble material obtained from phase I organisms was further treated to selectively remove either protein or carbohydrate. Protein was removed according to the method previously described (11) by using protease (Sigma), trichloroacetic acid, and ammonium sulfate. Carbohydrate was removed by placing the material into dialysis tubing and dialyzing against 0.1 N HCl for 48 h. The HCl breaks the α and β linkages of the carbohydrate, and the resulting monomers diffuse out of the tubing (4). Protein content of all soluble antigens (TCA-I, TCA-II, trichloroacetic acid-carbohydrate, and trichloroacetic acid-protein) was estimated by Δ absorbance at 224 and 233 nm (9), and carbohydrate was estimated by using the orcinol reaction (3).

Lymphocyte transformation assays. Lymphocyte transformation responses of human subjects to the various antigens described were evaluated by using the whole-blood culture technique of Han and Pauly (10). Whole blood obtained by venipuncture was diluted 1:30 in heparinized (10 U/ml of blood) RPMI-1640. Three-milliliter portions of the diluted blood were dispensed into disposable tissue culture tubes (no. 3033, Falcon Plastics) and either phytohemagglutinin-P (Difco) at a dilution of 1:100 or antigen added in 0.1-ml amounts. The cultures were incubated 120 h at 37 C in a 5% CO_2–95% air atmosphere. At the end of this time, 1.0 μCi of [H^3]thymidine (specific activity, 6.7 mCi/mmol) was added, and the cultures were incubated for an additional 18 h. The cultures were harvested by collecting the trichloroacetic acid-precipitable material onto glass fiber filters after lysis of erythrocytes by 3% acetic acid. The filters were placed in scintillation vials and dried, and 7 to 8 ml of scintillation fluid consisting of 2,5-diphenyloxazole and 1,4-bis-2-(5-phyloxazolyl)-benzene in toluene was added. Counts per minute were determined by using a Unilux III (Nuclear-Chicago) scintillation counter. Disintegrations per minute (dpm) were calculated from this data. Stimulation ratios were calculated by the following formula: stimulation ratio = (dpm of stimulated culture)/(dpm of control culture).

Serological procedures. The microagglutination test was performed by the procedure of Fiset et al. (8) to measure antibody titers in human subjects used in this study. The phase I antigen used was a formalin-killed suspension of purified organisms. The phase II antigen was prepared by treating purified phase I organisms with 0.5 N trichloroacetic acid. This resulted in a preparation with phase II antigenic characteristics but with none of the disadvantages of using native phase II organisms (8). All antibody titrations were carried out on sera treated at 56 C for 30 min.

Procedures performed before this study. All initial diagnostic procedures including complement fixation or radioisotope precipitation tests were performed on acute and convalescent serum samples by the Rocky Mountain Laboratory, Hamilton, Mont., during the period of 1966 to 1969.

Individuals were evaluated for possible vaccination against Q fever by skin testing with Q fever vaccine 58A, obtained from the Rocky Mountain Laboratory, as the skin test reagent. Those demonstrating negative reactions received one dose of the vaccine consisting of a 1.0-ml (10 colony-forming units) dose given subcutaneously. Skin testing and vaccination procedures were carried out by personnel at the Student Health Center, Washington State University, Pullman, Wash., during 1969 to 1970. The majority of the individuals who were subsequently vaccinated were not available at the time the current study was performed.

RESULTS

Chemical analyses of trichloroacetic acid-soluble fractions. Protein and carbohydrate determinations on the trichloroacetic acid-soluble phase I fraction revealed protein and carbohydrate concentrations similar to other reported

values and ranged from 200 to 400 μg of protein per ml and 300 to 700 μg of carbohydrate per ml, depending on the degree of concentration. Material obtained from phase II organisms under identical conditions also contained protein and carbohydrate but in much lower concentrations. Protein was found to range from 25 to 30 μg/ml and carbohydrate from 40 to 50 μg/ml. Efforts to rid the phase I trichloroacetic acid-soluble fraction of protein yielded preparations that were devoid of protein, as assayed by ultraviolet spectroscopy. Also, dilute acid treatment of the crude phase I fraction yielded carbohydrate-free fractions as monitored by the orcinol reaction. Neither procedure significantly altered the concentration of the remaining moiety.

Lymphocyte transformation response of individuals with varying histories of exposure to C. burneti. Individuals studied were grouped on the basis of their past history of exposure to *C. burneti*. Three individuals (E.H., C.H., and D.J.) had an illness with symptoms suggestive of Q fever and were subsequently serologically diagnosed as having been infected with *C. burneti* by the demonstration of either a rising complement fixation antibody titer or by using the Q radioisotope precipitation test. One individual (K.M.) had an illness that resembled Q fever in symptomology and response to antibiotics, but serological studies were not performed. Three individuals (T.J., L.M., and T.M.) had no past history of infection, nor were ever vaccinated, but worked with the organism. Another individual studied (B.F.) had no past history of exposure to *C. burneti* but, when skin tested prior to vaccination, demonstrated a borderline positive skin test and, consequently, did not receive the vaccine. One other individual (B.G.) demonstrated a negative skin test and received one dose of a phenol-formalin killed phase I preparation used as a vaccine. Also, individuals (G.B. and S.M.) with no past history of exposure or opportunity for recent exposure were specifically included in this study as negative controls. One individual (G.G.) originally bled as a negative control worked with *C. burneti* in a research problem and subsequently developed an illness suggestive of Q fever. The illness was confirmed as Q fever by a rising microagglutination titer. This individual was bled during the illness and at weekly intervals thereafter and studied by lymphocyte transformation and antibody titer.

The lymphocyte transformation responses of the individuals with histories of past exposure to *Coxiella burneti* are presented in Table 1. Also, the individuals used as negative controls are included. Antibody titers obtained at the time of initial lymphocyte transformation were determined by using the microagglutination procedure described and are included in Table 1.

The three individuals who were confirmed as having Q fever some years ago all responded to the whole-cell antigens (WCP-I and WCP-II) as well as the soluble phase I antigen (TCA-I) to a marked degree. Although all of these individuals demonstrated a diagnostic rise in antibody titer at the time of convalescence, none demonstrated any titer at the time lymphocyte transformation assays were performed.

The three individuals who had worked with live *C. burneti* (T.J., T.M., and L.M.) and the one individual with a past illness suggestive of Q fever (K.M.) also showed marked lymphocyte stimulation to the three antigens used. None of these individuals demonstrated an antibody titer at the time of testing.

The data obtained from the individuals evaluated for vaccination, one of which demonstrated a borderline positive skin test (B.F.) and one who was skin test negative and received one dose of the experimental vaccine (B.G.), and the responses of the two individuals used as negative controls are included in Table 1. None of these individuals responded to the antigens employed as determined by lymphocyte transformation nor did they demonstrate any detectable antibody titer.

The one individual (G.G.) originally bled as a negative control but who contracted Q fever during the course of this study was tested at weekly intervals after the actual illness (Table 2).

Individuals that responded to the trichloroacetic acid-extractable phase I antigen were further tested by using the two fractions of this antigen obtained by enzymatic and dilute acid hydrolysis. The responses obtained from these individuals to the protein and carbohydrate-free fractions are included in Table 3. Although all individuals included in this study were tested with the trichloroacetic acid-protein and trichloroacetic acid-carbohydrate antigens, only those individuals that responded to the crude TCA-I antigen are included in Table 3. All individuals with a negative response to the crude TCA-I antigen also showed no response to the trichloroacetic acid-protein and trichloroacetic acid-carbohydrate preparations. Those individuals responding to the TCA-I antigen failed to respond to the material obtained from phase II organisms that was obtained under identical conditions (TCA-II). Also, no response

TABLE 1. *Lymphocyte transformation response of individuals with various histories of exposure to Coxiella burneti*

Individual	Antigens				
	Control	PHA	WCP-I	WCP-II	TCA-I
E.H.[a]					
Average dpm[b]	2,969 ± 1,957	101,215 ± 50,764	41,558 ± 5,056	33,384 ± 8,892	32,801 ± 19,492
Stimulation ratio		34.1	14.0	11.2	11.0
Antibody titer[c]			0	0	
C.H.[a]					
Average dpm	2,966 ± 86	108,670 ± 32,104	28,253 ± 4,171	15,114 ± 1,285	17,161 ± 3,273
Stimulation ratio		36.6	9.5	5.1	5.8
Antibody titer			0	0	
D.J.[a]					
Average dpm	2,072 ± 1,184	273,912 ± 97,705	36,796 ± 16,501	31,734 ± 4,505	20,216 ± 2,988
Stimulation ratio		132.2	17.8	15.3	9.8
Antibody titer			0	0	
T.J.[d]					
Average dpm	3,022 ± 277	181,283 ± 19,823	21,160 ± 1,945	33,622 ± 13,559	15,175 ± 946
Stimulation ratio		60.0	7.0	11.1	5.0
Antibody titer			0	0	
T.M.[d]					
Average dpm	1,216 ± 616	142,698 ± 59,058	26,623 ± 4,937	33,334 ± 19,364	25,620 ± 12,931
Stimulation ratio		117.3	21.9	27.4	21.1
Antibody titer			0	0	
L.M.[d]					
Average dpm	6,345 ± 1,407	174,214 ± 47,931	19,868 ± 786	24,406 ± 4,094	8,818 ± 827
Stimulation ratio		27.5	3.1	3.8	1.4
Antibody titer			0	0	
K.M.[e]					
Average dpm	1,164 ± 93	175,663 ± 20,853	11,240 ± 1,272	10,909 ± 128	15,569 ± 2,084
Stimulation ratio		151.0	9.7	9.4	13.4
Antibody titer			0	0	
B.G.[f]					
Average dpm	4,244 ± 1,974	146,773 ± 6,414	5,927 ± 1,684	7,922 ± 1,724	3,823 ± 576
Stimulation ratio		34.5	1.4	1.9	.90
Antibody titer			0	0	
B.F.[g]					
Average dpm	2,728 ± 588	152,606 ± 8,203	2,868 ± 384	5,786 ± 733	4,342 ± 975
Stimulation ratio		55.9	1.05	2.1	1.6
Antibody titer			0	0	
G.B.[h]					
Average dpm	4,447 ± 685	128,130 ± 6,066	3,400 ± 202	6,450 ± 1,296	1,846 ± 411
Stimulation ratio		28.8	.76	1.5	.42
Antibody titer			0	0	
S.M.[h]					
Average dpm	2,964 ± 257	107,000 ± 9,405	2,124 ± 725	2,091 ± 77	3,257 ± 674
Stimulation ratio		36.1	.72	.71	1.1
Antibody titer			0	0	

[a] These individuals had been serologically confirmed as having Q fever.
[b] Average response of two separate determinations.
[c] Determined by the microagglutination technique at the time of lymphocyte transformation assay.
[d] These individuals worked with live *C. burneti* in laboratory situations.
[e] This individual had an illness suggestive of Q fever in symptomology and response to antibiotics but serological studies were not performed.
[f] Received an experimental vaccine 4 years previously.
[g] Was skin test borderline when evaluated for possible vaccination 4 years previously.
[h] Individuals with no past history of exposure to *C. burneti* included as negative controls.

TABLE 2. *Lymphocyte transformation and antibody response of an individual (G.G.) who acquired Q fever during study*[a]

Date	Antigens				
	Control	PHA	WCP-I	WCP-II	Trichloroacetic acid-I
16 March 1974					
Average dpm[b]	3,965 ± 739	156,588 ± 35,440	5,612 ± 626	4,296 ± 142	1,624 ± 184
Stimulation ratio		39.5	1.4	1.1	.40
Antibody titer[c]			0	0	
5 April 1974					
Average dpm	1,182 ± 96	153,929 ± 26,604	1,617 ± 84	1,920 ± 121	942 ± 106
Stimulation ratio		130.2	1.4	1.6	.80
Antibody titer			0	16	
10 April 1974					
Average dpm	982 ± 37	149,922 ± 15,204	178,001 ± 14,753	98,720 ± 10,639	70,896 ± 1,546
Stimulation ratio		152.7	181.3	100.5	72.2
Antibody titer			16	32	
17 April 1974					
Average dpm	1,224 ± 55	106,274 ± 6,191	49,023 ± 3,148	46,005 ± 3,114	15,459 ± 2,314
Stimulation ratio		86.8	40.1	37.6	12.6
Antibody titer			16	32	
13 Sept 1974					
Average dpm	903 ± 128	206,521 ± 11,740	106,657 ± 15,105	33,070 ± 2,293	9,279 ± 294
Stimulation ratio		228.7	118.1	36.6	10.3
Antibody titer			8	0	

[a] Onset of symptoms occurred on 1 April 1974 and individual was released from hospital on 6 April 1974.
[b] Average of duplicate cultures.
[c] Determined by the microagglutination technique at time of lymphocyte transformation assay.

TABLE 3. *Lymphocyte transformation response to trichloroacetic acid-soluble material obtained from phase I and phase II organisms and fractions of the phase I trichloroacetic acid-soluble material*

Individual	Antigens				
	Control	TCA-I	TCA-II	TCA-C	TCA-P
E.H.					
Average dpm[a]	2,969 ± 1,957	32,801 ± 19,492	3,896 ± 2,206	2,502 ± 2,250	31,549 ± 10,684
Stimulation ratio		11.0	1.3	.84	10.6
C.H.					
Average dpm	2,966 ± 86	17,161 ± 3,273	2,115 ± 253	2,315 ± 148	18,002 ± 3,494
Stimulation ratio		5.8	.71	.78	6.1
D.J.					
Average dpm	2,072 ± 1,184	20,216 ± 2,988	2,727 ± 442	2,095 ± 1,204	9,059 ± 1,472
Stimulation ratio		9.8	1.3	1.0	4.4
T.J.					
Average dpm	3,022 ± 277	15,175 ± 946	2,550 ± 1,953	2,715 ± 1,050	9,935 ± 1,304
Stimulation ratio		5.0	.84	.90	3.3
T.M.					
Average dpm	1,216 ± 616	25,620 ± 12,931	1,223 ± 1,130	1,479 ± 876	21,456 ± 17,073
Stimulation ratio		21.1	1.0	1.2	17.6
K.M.					
Average dpm	1,164 ± 93	15,569 ± 2,084	1,235 ± 275	1,350 ± 357	12,669 ± 4,184
Stimulation ratio		13.4	1.1	1.2	10.9

[a] Average of two separate determinations.

was noted to the phase I trichloroacetic acid-soluble material that was treated to remove the protein component (tricholoracetic acid-carbohydrate). In contrast, all individuals responding to the TCA-I antigen also responded to the material selectively treated to remove the carbohydrate component leaving only the protein (trichloroacetic acid-protein).

DISCUSSION

A state of delayed hypersensitivity has been shown to develop after vaccination or infection with *C. burneti* (11). In humans, this hypersensitivity has been demonstrated by skin test reactivity but due to the inherent toxicity of many of the rickettsial antigen preparations (2), interpretation of results has been difficult. In studies employing guinea pigs, the existence of delayed hypersensitivity after infection with *C. burneti* was demonstrated by using inhibition of macrophage migration as the criterion of sensitivity (11). The development of delayed hypersensitivity after exposure to *C. burneti* undoubtedly implies the concomitant establishment of an active cell-mediated immune response that may play an important role in combating Q fever.

It is apparent from the individuals studied in this report that antigen recognition, at the cellular level, develops after Q fever infections. All individuals with proven past infections responded to the whole-cell, *C. burneti* antigens as determined by in vitro lymphocyte transformation. All of these individuals had demonstrated a diagnostic antibody titer, determined by complement fixation or radioisotope precipitation techniques, after convalescence. However, no antibody titer was detectable at the time these individuals were tested for their specific lymphocyte transformation response. These data would suggest that although the antibody response drops to a level not detectable by the microagglutination test, cells still remain in the peripheral circulation that respond to specific antigen stimulation. The studies of the one individual (G.G.) who became ill after exposure to the organism indicate the value of this technique as an indicator of responsiveness. Before exposure, no response could be elicited by the antigens nor was any response noted during the acute phase of the illness. The results obtained from this individual, when combined with the results obtained from individuals infected up to 8 years ago, would suggest that in vitro responsiveness to the antigens employed, as measured by lymphocyte transformation, develops in a short time after recovery and remains at a demonstrable level for a substantial period of time. This study clearly demonstrates that the cell-mediated immunity present after infection by *C. burneti* may be successfully determined by ascertaining the lymphocyte transformation response.

The response to the fraction obtained by trichloroacetic acid extraction of phase I cells indicates that this material contains an antigenic component(s) capable of stimulating immune lymphocytes. In contrast, the trichloroacetic acid-soluble fraction obtained from phase II organisms was not stimulatory, which suggests that the necessary antigenic component(s) is either absent or present only in small amounts in these organisms.

Selective removal of the protein portion of the trichloroacetic acid fraction (phase I) by enzymatic treatment resulted in a preparation which did not elicit a lymphocyte response. This would appear to correlate with previous reports that protein denaturation of the trichloroacetic acid fraction by phenol treatment results in an antigenically inactive preparation (1). The observation that removal of the carbohydrate moiety did not alter the stimulatory ability of the preparation is somewhat surprising when considered along with other reports (11). This phenomenon may be explained by the presence of carbohydrate remaining below the limits of detection of the assay procedure employed. It is also possible that the carbohydrate moiety is not a major factor in the phase variation of *C. burneti*. This latter possibility does not seem highly plausible, however, when cell envelope composition data of phase I and phase II organisms are considered (T. R. Jerrells, D. J. Hinrichs, and L. P. Mallavia, Can. J. Microbiol., in press). The carbohydrate nature of this antigen has been further suggested by the work of Ormsbee (17). Although definitive experiments are lacking, it is possible that the active species in the trichloroacetic acid-soluble fraction of *C. burneti* phase I is a protein-carbohydrate complex and that the response to the protein portion is a carrier-specific response.

Also of interest was the apparent lack of responsiveness of the single individual who had received one injection of the experimental vaccine. Previous studies have demonstrated that specific lymphocyte responsiveness to *Rickettsia* sp. antigens occurred only after a second injection of the killed vaccine and even under these conditions not all individuals became responsive (5). These findings suggest that if cell-mediated immunity is responsible for protection, the experimental vaccine which was

used with the one individual available for study produces either a short-lived immunity or at best a minimal immune state. The high level of responsiveness noted in individuals that had encountered living organisms suggests that long-lived immunity to *C. burneti* may require the presence of living organisms or repeated exposure to killed organisms.

ACKNOWLEDGMENTS

We thank the subjects used in this study for their participation and patience and Janice Jerrells for her assistance in the preparation and typing of the manuscript.

This research was supported in part by U.S. Army Research and Development Contract DADA17-73-C-3090.

LITERATURE CITED

1. Anacker, R. L., W. T. Haskins, D. B. Lackman, E. Ribi, and E. G. Pickens. 1963. Conversion of the phase I antigen of *Coxiella burnetii* to hapten by phenol treatment. J. Bacteriol. **85**:1165-1170.
2. Anacker, R. L., D. B. Lackman, E. G. Pickens, and E. Ribi. 1962. Antigenic and skin-reactive properties of fractions of *Coxiella burnetii*. J. Immunol. **89**:145-153.
3. Ashwell, G. 1957. Colorimetric analysis of sugars, p. 73-105. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
4. Clark, J. M. (ed.). 1964. Experimental biochemistry, p. 4-18. W. H. Freeman and Co., San Francisco.
5. Coonrod, J. D., and C. C. Shepard. 1971. Lymphocyte transformation in rickettsioses. J. Immunol. **106**:209-216.
6. Cracea, E., R. Voiculescu, G. Zarnea, and M. Ionescu. 1970. Electron microscopic study of phase I and phase II *C. burneti* in the chick yolk sac by use of ferritin conjugated antibody. Z. Immunitaetsforsch. Allerg. Klin. Immunol. **140**:358-365.
7. Fiset, P. 1957. Phase variation of *Rickettsia* (*Coxiella*) *burneti:* study of the antibody response in guinea pigs and rabbits. Can. J. Microbiol. **3**:435-445.
8. Fiset, P., R. A. Ormsbee, R. Silberman, M. Peacock, and S. H. Spielman. 1969. A microagglutination technique for detection and measurement of rickettsial antibodies. Acta Virol. Engl. Ed. **13**:60-66.
9. Groves, W. E., F. C. Davis, Jr., and B. H. Sells. 1968. Spectrophotometric determination of microgram quantities of protein without nucleic acid interference. Anal. Biochem. **22**:195-210.
10. Han, T., and J. Pauly. 1972. Simplified whole blood method for evaluating *in vitro* lymphocyte reactivity of laboratory animals. Clin. Exp. Immunol. **11**:137-142.
11. Heggers, J. P., L. P. Mallavia, and D. J. Hinrichs. 1974. The cellular immune response to antigens of *Coxiella burneti*. Can. J. Microbiol. **20**:657-662.
12. Hoyer, B. H., R. A. Ormsbee, P. Fiset, and D. B. Lackman. 1963. Differentiation of phase I and phase II *Coxiella burnetii* by equilibrium density gradient sedimentation. Nature (London) **197**:573-574.
13. Lackman, D. B., E. J. Bell, J. F. Bell, and E. G. Pickens. 1962. Intradermal sensitivity testing in man with a purified vaccine for Q fever. Am. J. Public Health **52**:87-93.
14. Lennette, E. H., W. H. Clark, F. W. Jensen, and C. J. Toomb. 1952. Q fever studies. XV. Development and persistence in man of complement-fixing and agglutinating antibodies to *Coxiella burnetii*. J. Immunol. **68**:591-598.
15. Luoto, L., J. F. Bell, M. Casey, and D. B. Lackman. 1963. Q fever vaccination of human volunteers. 1. The serologic and skin-test response following subcutaneous injections. Am. J. Hyg. **78**:1-15.
16. Ormsbee, R. A. 1962. A method of purifying *Coxiella burnetii* and other pathogenic rickettsiae. J. Immunol. **88**:100-108.
17. Ormsbee, R. A., E. J. Bell, and D. B. Lackman. 1962. Antigens of *Coxiella burnetii*. I. Extraction of antigens with nonaqueous organic solvents. J. Immunol. **88**:741-749.
18. Ormsbee, R. A., M. Peacock, G. Tallent, and J. J. Munoz. 1968. An analysis of the immune response to rickettsial antigens in the guinea pig. Acta Virol. Engl. Ed. **12**:78-82.
19. Stocker, M. G. P., and P. Fiset. 1956. Phase variation of the nine mile and other strains of *Rickettsia burneti*. Can. J. Microbiol. **2**:310-321.