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STUDIES ON THE ANTIGENIC COMPOSITION OF 'COXIELLA BURNETII'.(U)
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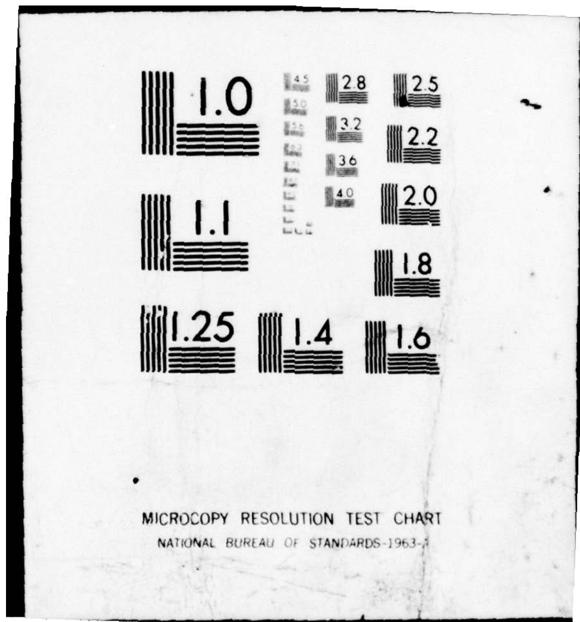
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The in vitro resistance of mice to infection with C. burnetii was evaluated using various immunosuppressive agents. Spleen impressions of control mice indicated that maximal infection occurred during the first week, after which time C. burnetii was rapidly cleared from the spleen. Treatment of mice prior to C. burnetii infection with ALS, AMS, or silica (i.p.) failed to cause lethal infection, however, spleen impressions from these treated animals demonstrated that clearance of rickettsiae from the spleen occurred at a much slower rate when

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20. compared to control infected animals over the same time period. Cyclophosphamide treatment prior to infection was found to cause 100% mortality within 10 days post infection. When silica was administerd 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.

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Studies on the Antigenic Composition of *Coxiella burnetii*

Summary

Soluble antigen preparations of *Coxiella burnetii* contain mitogenic and endotoxic properties. The rickettsial derived material commonly referred to as "Phase I antigen" causes a blastogenic response in mouse lymphocyte cultures. On a comparative level this trichloroacetic acid extracted material has about 25% of the mitogenic activity as does purified *E. coli* lipopolysaccharide. The mitogenic activity of the *C. burnetii* material has its direct effect on mouse B lymphocytes. The material does bind to all lymphocytes (demonstrably in vitro) and promotes increased levels of T lymphocyte activity when combined with T lymphocyte mitogens. This in vitro activity has, as yet, not been shown to correlate with the in vivo events that occur in the normal or athymic mouse following infection with *C. burnetii*. No apparent non-specific activation occurs as judged by immunosuppressive studies. These studies suggest that the mechanism of resistance to *C. burnetii* in the adult mouse is one of an acquired cellular response and the innate mitogenic activity of *C. burnetii* derived antigensdoesnot contribute to a non-specific event.

Studies relating to adjuvant augmentation of the specific cellular response were not successful in terms of expectations.

Progress to Date

Our purpose during the time period coverd by this annual report was to examine techniques designed to produce specific cell-mediated immunologic adjuvants and to selectively measure aspects of the biologic effects of antigen preparations of *C. burnetii*. This latter study also led us into an investigation of the mechanism of *C. burnetii* resistance in the mouse.

The studies of adjuvants designed to augment specific cell-mediated immunity were brought about by a series of recently published reports (J. of IMMUNOLOGY 114:1518, 1975). These reports dealt with the adjuvant properties of fatty acid conjugated protein antigens. These preparations were reportedly effective in elicting an antigen specific cell-mediated response. Because of the importance of the cellular immune response in combating intracellular parasites we felt that this new procedure warranted further evaluation. We carried out our studies in the guinea pig system and employed dinitrophenylated as well as unconjugated purified serum proteins as antigens. We conjugated these antigen preparations with molar excesses of dodecanoic anhydride in order to achieve suitable conjugation ratios of the fatty acid. Guinea pigs were immunized with these antigen preparations and evaluated immunologically by skin test response as well as by lymphocyte transformation and migration inhibition assays. In all experiments carried out to date less than 20% of the animals responded with a significant cell-mediated response.

The procedure of conjugation may have some empirical aspects that we have not determined and the affect of the fatty acid conjugation on reactivity has not been chemically defined. The role of fats and lipids in immune recognition is an important area for future development but the current state of the art does not apparently allow direct application. Our efforts in this regard did not meet with sufficient success to continually investigate this interesting problem and our attention has been re-focused within the C. burnetii system.

Our studies of the relationship between antigen-mitogen-immunogen in the C. burnetii system are continuing and have yielded some interesting information. We have initially examined the above relationship for the TCA extracted Phase I antigen of C. burnetii. Although this antigenic complex may not be the immunogen of choice we choose to start with this material since it has been worked with extensively and is both antigen, mitogen and immunogen. The antigenic property, defined

by a cell-mediated response, is associated with the protein component. Although an immune response can be generated by this preparation the relationship of this response to protection has as yet not been tested. We have defined, in vitro, the mitogenic property of this preparation in lymphocyte cultures. Mouse spleen lymphocyte cultures respond to the presence of the TCA antigen by undergoing classic transformation. We feel this response is exclusively a B-lymphocyte response since cultures depleted of macrophages respond to higher levels than do unpurified cell populations. Also, the response of the athymic mouse is of the same order of magnitude as the normal mouse. This activity may relate to our findings of similar levels of antibody formation to _C. burnetii_ in nude, normal and some immunosuppressed mice (see below). The mitogenic activity of the TCA extract is only apparent in mouse lymphocyte cultures. Other systems (guinea pig, rabbit, human) do not reflect this B-lymphocyte mitogenicity. However, in these systems, as in the mouse, T-lymphocyte TCA binding does apparently occur. In this regard we have conducted experiments in which we have evaluated the dose dependent response of Phytohemagglutinin and Concanavalin A. These studies demonstrated that the threshold level of T lymphocyte mitogen needed to stimulate a response in vitro was lowered by the presence of the _C. burnetii_ derived material. Furthermore, at T mitogen levels in which substancial cell activity could be detected, the presence of the TCA extract [illegible] increased this activity. This latter observation [illegible] also indicate that the B-lymphocyte (not mouse) needs a double signal for activation and that the activated T lymphocyte provides that second signal. The TCA product is of course a complex extract. The lipid contribution is in evidence and may explain the mitogenic event. The material is still quite toxic as determined by physical response following injection or by in vitro assay. Other chemical

preparations are in the process of being defined and will be the subject of future communications when coupled with in vivo evaluations of the preparations as protective immunogens.

We have also completed a study of the in vivo response of neonate, athymic and normal laboratory mice to C. burnetii. The study followed from our observations of the effects of C. burnetii antigen derivatives on mouse lymphocytes and from our previous studies which showed that the mouse macrophage resisted the intracellular parasitism of C. burnetii. We are also aware of other reports that mice are resistant to infection with C. burnetii.

In order to evaluate the mechanism of resistance in the mouse to C. burnetii infection we initially compared in vivo clearance of the rickettsia in normal and immunosuppressed mice. The effect of the various immunosuppressive agents that we employed in this study are shown in Table I. Animals pretreated with 6 mg of cyclophosphamide intraperitoneally 24 h prior to infection with C. burnetii showed a high degree of susceptibility to the organism. Mortality was first observed at day 6 with deaths occurring up through day 10 post-infection by which time 100 % of the infected animals had died. No mortality occurred in the control groups which received cyclophosphamide or C. burnetii only. Upon death of the animals spleen impressions were examined and found to contain large numbers of rickettsial organisms as shown in Table 1.

Animals pretreated with 0,5 ml of anti-lymphocyte or anti-macrophage serum 24 h prior and 24 h post-infection with C. burnetii demonstrated no mortality. They were sacrificed at 7 day intervals and the fate of the injected rickettsiae was noted by spleen impressions. The results presented in Table 1 suggest that both ALS and AMS treatment hinder rickettsial clearance from the spleens of infected animals as compared to the control animals.

Silica treatment was carried out using two routes of injection. One group of animals received 50 mg of silica

Table I. Effect of various agents on the in vivo response of Swiss-Webster mice to infection with C. burnetii, Phase I.

Days post infection[a]	Treatment[b]	Antibody titer[c]	Spleen impression smear evaluation[d]
7	Control infected	32	3+ (2+-4+)
	Cyclophosphamide infected[e]	32	4+ (3+-4+)
	Antilymphocyte serum infected	16	4+ (2+-4+)
	Antimacrophage serum infected	32	3+ (2+-3+)
	Silica (i.p.) infected	16	3+ (3+-4+)
	Silica (i.v.) infected	32	4+ (All)
14	Control infected	128	1+ (1+-2+)
	Antilymphocyte serum infected	128	3+ (2+-3+)
	Antimacrophage serum infected	128	3+ (2+-3+)
	Silica (i.p.) infected	256	2+ (1+-3+)
	Silica (i.v.) infected	64	4+ (3+-4+)
21	Control infected	256	± (Neg-1+)
	Antilymphocyte serum infected	512	1+ (±-2+)
	Antimacrophage serum infected	256	1+ (1+-2+)
	Silica (i.p.) infected	128	1+ (±-1+)
	Silica (i.v.) infected	512	Neg (Neg-±)

[a] Animals were infected with 0.1 ml of C. burnetii intravenously (containing 10^6 egg LD_{50}/ml).

[b] See materials and methods for dosage and schedule of the various treatments.

[c] Determined using the microagglutination technique.

[d] Graded according to the following protocol: Negative - no rickettsiae detected; ± - rare rickettsiae noted; 1+ - occasional cell noted containing greater than 20 rickettsiae; 2+ - cells containing greater than 20 rickettsiae in majority of fields; 3+ - cells containing greater than 20 rickettsiae in all fields; 4+ - cells containing greater than 20 rickettsiae in all fields and many free rickettsiae noted. Numbers in parenthesis represent range of grades noted.

[e] All of cyclophosphamide treated infected animals died of rickettsial infection by day 10 post infection.

intraperitoneally while a second group of animals received 3 mg of silica intravenously. Both treatments were performed 24 h prior to infection with *C. burnetii*. Animals receiving silica intravenously showed 50% mortality with deaths occurring during the second week of infection. Animals receiving intraperitoneal injections of silica showed no mortality after infection with *C. burnetii*. The results in Table 1 show that silica treatment is similar to the other immunosuppressive agents in that it reduces the rate of clearance of *C. burnetii* as compared to control animals. It should also be noted that silica administered intravenously was much more efficient in its suppressive action than intraperitoneal treatment.

Also presented in Table 1 are the antibody titers for each treatment group of animals. None of the treatments was capable of eliminating the antibody response to *C. burnetii*. Since cyclophosphamide treated animals all died by day 10 post-infection, experiments were initiated using smaller infecting doses of rickettsia in an attempt to prolong survival. Animals surviving through the second week post-infection were bled and antibody titers determined. Antibody titers of the treated and control infected groups were not significantly different which would indicate that cyclophosphamide does not eliminate the production of antibody to *C. burnetii*.

To determine if the rickettsiae visualized in spleen impressions were viable, dilutions of spleen homogenates were injected into 6 day old embryonated eggs. At the termination of the experiment, smears were made from the yolk sacs and evaluated for the presence of rickettsiae. Table II presents the percentage of yolk sacs infected from each dilution of the various spleen homogenates. As can be seen, spleens obtained from infected control animals contain viable rickettsiae at 7 days post infection. Animals pretreated with ALS, AMS, silica, or cyclophosphamide also contain viable rickettsiae, but based

Table II. Viability of C. burnetii in spleen homogenates of treated mice as assayed by egg infectivity.[a]

Type of treatment[b]	Dilution of homogenate[c]	Days post infection[d] 7	14
None	10^{-1}	100[e]	20
	10^{-2}	0	0
	10^{-3}	0	0
ALS	10^{-1}	100	33
	10^{-2}	67	33
	10^{-3}	50	0
AMS	10^{-1}	100	75
	10^{-2}	100	33
	10^{-3}	50	0
Silica (i.p.)	10^{-1}	100	50
	10^{-2}	33	25
	10^{-3}	33	0
Silica (i.v.)	10^{-1}	100	50
	10^{-2}	75	20
	10^{-3}	66	0
Cyclophosphamide	10^{-1}	100	ND[f]
	10^{-2}	100	ND
	10^{-3}	33	ND

[a] See materials and methods.

[b] All treatments were administered 24 h prior to infection with C. burnetii except ALS and AMS which were administered 24 h prior and 24 h post infection.

[c] Each dilution of the spleen homogenate was administered in 0.1 ml volumes.

[d] Spleens were collected from infected mice at these time periods after infection with C. burnetii.

[e] Numbers represent the percent death of surviving eggs after 48 h to allow for death due to trauma occurring during inoculation.

[f] ND - Not done due to death occurring before 14 days of cyclophosphamide treated mice.

on the dilution used, the immunosuppressed animals contained more viable organisms than normal animals. The data obtained using spleens from animals obtained 14 days post infection suggest that the immunosuppressed animals retain viable rickettsiae for greater time periods than normal animals. Spleens obtained 21 days post-infection were uniformly negative in their ability to transfer rickettsiae; thus, the immunosuppressive effects of the various agents, as administerd, are transitory.

This study implies that cell-mediated immunity is important in establishing resistance to C. burnetii in the mouse. Lymphocytes and macrophages are both important in control as seen from the effects of cyclophosphamide as well as silica. Perhaps in this system, as in other diseases controlled by the cell-mediated immune system, there exists a lymphocyte macrophage cooperation. It is apparent from the AMS and silica treated animals that the natural resistance of the mouse to infection by C. burnetii involves the macrophage directly, and the normal mouse macrophage may retard intracellular replication of C. burnetii only for a limited period of time. However, this initial delay of rickettsial replication allows time for the necessary lymphocyte mediated events to occur, after which time an effective macrophage function ensues. Thus, it would appear that mouse resistance may in reality be an acquired cell-mediated event resembling other well studied organisms such as L. monocytogenes and M. tuberculosis.

Further support for this concept of cellular resistance can be seen from our observations of C. burnetii in athymic and neonate mice. The athymic and young mouse less than 2 weeks of age can not resist a C. burnetii challenge and eventually die from the infection. Death occurs in the athymic mouse even though substantial levels of circulating antibody are in evidence. In the young mouse the observations would indicate that the lack of a developed T-lymphocyte population is critical and that cellular

cooperation is a necessity for control of _C. burnetii_.

The observations in the mouse indicate that it may be a suitable species for the study of _C. burnetii_ infection and the associated immune response. The initial organ involvement and cellular basis for resistance are evidence for an initial establishment of the parasite in vivo and secondarily an accelerated clearance that has a recognized immunologic basis.

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