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Enhancement of Ehrlichia canis Infection of Canine Blood Monocytes in vitro through the addition of Seropositive and Monoclonal Antibody

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Enhancement of *Ehrlichia canis* Infection of Canine Blood Monocytes *in vitro* through the addition of Seropositive and Monoclonal Antibody

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Abstract

Ordinary immune response theory dictates that the addition of specific antibody to an infected culture should neutralize the antigen present. Preliminary experimentation indicated that *Ehrlichia canis* defies this pattern in cultures of dog bone marrow cells. To confirm these initial findings two experiments were conducted. Experiment 1 sought to prove that the addition of dog serum positive for *E. canis* to a culture of dog bone marrow cells newly infected with *E. canis* would increase both the rate and degree of infection. The purpose of Experiment 2 was to attempt the same procedure using monoclonal rather than polyvalent antibody in order to produce a patentable culture technique and check for site specificity. The results of Experiment 1 show a dramatic increase in rate and degree of infection with the addition of positive serum, particularly up to the day seven mark. Experiment 2 did not show a high level of difference between IgG and IgM antibodies suggesting that the enhancement is not site specific. It is thought that this phenomena is related to the fact that *E. canis* targets canine blood monocytes, which are the very cells being called for in the humoral immune response. Further tests, primarily including PCR, would need to be run to attempt a quantification of the results before a definitive culture technique could be developed.

Introduction and Literature Review

Overview

Ehrlichia canis (*E. canis*) was first discovered in Algeria in 1935 and was noted in the Middle East and Eastern Asia soon after. The organism was first found in the United States in 1962 and due to the loss of hundreds of military German Shepherds infected with *E. canis* during Vietnam, many U.S. scientists set out to research the organism.[3] Characteristically, the *E. canis* bacterium belongs to the Family Rickettsiaceae, which also includes Chlamydial bacteria. *E. canis* causes a disease called canine Ehrlichiosis in both wild and domestic canines. The primary vector for the organism is *Rhipicephalus sanguineus* (the common dog tick), but has been shown to be carried occasionally by other members of the tick line. The Rickettsiae are obligate intracellular parasites feeding primarily on leukocytes within their host. The primary cell line for *E. canis* is the canine blood monocyte; as a result of this the cells are cultured experimentally in dog bone marrow cells (DBMs). *E. canis* is recognizable among the other Rickettsial bacteria because of its very loosely packed cytoplasmic DNA, and can be noted in a cell line by its densely packed morulae (> 100 organisms per vacuole).[3,10] *E. canis* has become a matter of particular interest in the last decade because of its close similarity to the bacteria that causes human Ehrlichiosis. *E. canis* antibodies are actually used effectively to test for human Ehrlichiosis, as no human isolate has been found as of yet.[8]

Pathogenesis

The incubation period for canine Ehrlichiosis is one to three weeks. Symptoms of the disease include fever, depression, anorexia, encephalitis, leukopoenia and thrombocytopenia. The canine can deal with low levels of infection fairly effectively by its natural defenses, it is only at higher levels of infection that disease is brought on. Canine Ehrlichiosis is predominately site specific affecting the lungs, kidneys and meninges.[4] Cells are cultured as mentioned above in dog bone marrow cells. The typical media used is Fischer's media + $NaHCO_3$ + hydrocortisone + L-glutamate + 20% horse serum; this media keeps cells in the proper pH range and ensures they have enough CO_2 for respiration. Cells must however be passed twice a week to replace the nutrients depleted in media. Flasks infected with Ehrlichia can be distinguished from other bacteria by the lack of a plaque formation on the monolayer of host cells in the flask and can be recognized among non-infected flasks because infected cells detach from the monolayer and float. This release of Ehrlichial cells is not only from cell lysis, but also from cellular exocytosis.[10, 11]

The most critical symptoms of canine Ehrlichiosis are platelet disorders and serum protein alterations due to their close relation to the immune system. Thrombocytopenia and anti-platelet antibodies (APA) are the two most prevalent platelet disorders. Thrombocytopenia (low platelet count) is the most common hematological abnormality caused by *E. canis* infection. It is attributed to increased platelet consumption during infection, increased splenic sequestration of platelets and a decrease in platelet life span; all of these occurring during the acute stage of infection. The presence of serum platelet-bindable APA indicates that an immune disruption probably also contributes to

thrombocytopenia. The appearance of APA prior to detection of Ehrlichial infection suggests an increase in B cell count (B cells carrying natural autoantibody receptors), which implies that Ehrlichial antigens are interacting with and disrupting the immune system even prior to the development of detectable *E. canis* antibodies.[3]

The other major symptomal disorder is serum protein alterations. The alterations present occur in the form of hyperglobulinemia, hypergammaglobulinemia and hypoalbuminemia. Hyperglobulinemia is characterized by α_2 and β_2 –globulin increase. This increase is thought to be in response to tissue damage and inflammation. Hypergammaglobulinemia is attributed to an exaggerated immune response to the *E. canis* antigen, so much exaggerated that the antibodies produced are nonspecific and not even effective against *E. canis*. The over presence of these polyclonal nonspecific antibodies is the source of the excess of gamma globulin, not the actual *E. canis* antibodies themselves. The hypoalbuminemia is thought to be an indirect compensation effect of the hypergammaglobulinemia in order to maintain oncotic pressure levels.[3]

Commonly effective treatments include oxytetracycline and doxycycline, and both eliminate carrier status of the infection. Although the bactaremic phase is halted, the immune response effects are long lasting.[3, 10] It has actually been shown that dogs may remain carriers in excess of 34 months past their clinical stage, even with effective treatment.[2]

Immune Response

All Ehrlichial infections induce an immune response regardless of clinical signs or detectable presence of antibodies. Lasting effects of *E. canis* are primarily characterized by parasitemia, even long after bactaremic infection and treatment. High antibody titers

following treatment and elimination of *E. canis* are of no protective value when the dog is again challenged with the bacteria. This suggests that there are no benefits from the canine humoral (B-cell) immune response. This paper actually proposes that the humoral response contributes to the pathogenesis of the disease in some way. Cellular (T cell) immune response appears then to be the only means of any protective immunity for the cell.[3, 6]

Isolation and Detection

E. canis cell lines are isolated from an inoculation of DBM cell culture from an infected buffy-coat, containing mononuclear cell fractions. Cultured cell lines behave similarly to *in vivo* behavior, with very few exceptions. The most notable being that some mutation occurs in the culture cell line affecting vector capability. Dogs infected with a cell culture strain of *E. canis* rather than a wild strain will not transfer infection to other dogs through tick bites. For some, as of yet unknown, reason, the tick does not transmit the cultured strain of *E. canis* as it would a wild strain.[1]

Detection of the organism can be made by a variety of tests. A direct blood smear with a gram stain of the peripheral buffy coat will show pink (Gram -) if the organism is present, but this test alone is obviously inconclusive. An indirect immunoflourescent antibody test (IFA) is the most common means of diagnosis.[10] The IFA involves a sample of antigen being treated with a primary and conjugate antibody to test for infection. The primary antibody in the case of *E. canis* antigen is serum from an Ehrlichial-infected canine macrophage. The conjugate is a flourescine-labled goat-anti-dog or rabbit-anti-dog. If the *E. canis* antigen is present in the sample, the primary antibody will bond to it and the conjugate antibody will bond to the primary. The

flourescine base of the conjugate antibody can be detected under a fluorescent microscope.[1]

Another method of detection, which is becoming increasingly popular, is polymerase chain reaction (PCR) testing. A PCR based detection assay specifically detecting the presence of *E. canis* in the acute stage of infection was first reported by McBride et al. in 1996.[7] The need for a PCR method of detection was evident in the ineffectiveness of other detection methods. Direct blood smear was usable, but Ehrlichia is very difficult to find in any one-sample smear of blood, particularly due to the site-specificity of the organism. As a result of this, false-negative smears are common. IFA testing is much more effective than direct blood smears, but still has its limitations. The primary limitation is that IgM and IgG antibodies, which are the primary antibodies present in Ehrlichial infection, are not detectably present until one to three weeks post infection. Additionally, there is a great deal of cross-reactivity between Ehrlichial species; as is evidenced by *E. canis* antibodies being used to test for human Ehrlichiosis by IFA. Direct cell culture isolation of the organism is both sensitive and specific, but the process takes one to four weeks. The length of time necessary for the proper IFA detection and the cell culture isolation are the primary concerns here. Studies show that dogs diagnosed while still in the acute phase of infection have a very good prognosis for recovery if treatment is begun immediately. However, if *E. canis* is not diagnosed until the chronic stage, the prognosis for the dog is very poor, even with treatment. As a result of this need for early identification of the organism, a method for PCR testing was developed.[3, 7]

PCR involves the isolation of DNA, RNA or cDNA from a blood or tissue sample. The Nucleic Acid is then combined with a series of buffers, amplification primers and taq-polymerase and is run through a thermacycler for amplification. The primers are specific to the organism being tested for and are selected in conjugate pair to the gene sequence of the organism (usually ≈20 base pairs). The primers, along with the specific set of cycled temperatures in the thermacycler, ensure that only the targeted DNA of the desired organism will be amplified in the PCR process. If the original sample contained any trace of the organism, then its, and only its, DNA will be amplified by the primer sets in the PCR. Once the sample has been extracted and amplified, the amplicon can be tested using gel electrophoresis (an agarose gel with an ethydium bromide label in this case) to determine if any DNA, RNA of cDNA are present. If any nucleic acids show up in the gel, then the original sample contained the targeted organism. PCR is a much more effective technique for diagnosis, because it is independent of the production of antibodies to the organism present. PCR can detect the organism if even a trace of its DNA is present anywhere in the blood stream or in a sampled tissue rather than waiting on the organism to elicit an immune response and antibodies to be produced. The test itself can be conducted in less than a day and has been shown effective for diagnosis at less than one-week post infection. It also allows specific detection of the organism as there is no cross reactivity between nucleotides as there is with antibodies. It may also enable us to distinguish between seropositive infected and seropositive non-infected dogs.[2, 5, 7]

PCR technology has also made possible the cloning of specific genes of *E. canis* that are becoming crucially important in the understanding of the organism. McBride et al.

and Xue-Jie Yu et al. have isolated, cloned and characterized two genes responsible for the expression of the 28 and 120 Kilodalton (kDa) proteins respectively, which are proving to be useful in serodiagnosis of not only *E. canis*, but also other Ehrlichial species. These genes could prove to be the common link between the species and would thus greatly increase our understanding and ability to control the human Ehrlichial strain in the future.[9, 12]

Preliminary Experimentation

In the Spring of 1999 an experiment was conducted in the lab of Dr. Richard Corstvet at the Louisiana State University Department of Veterinary Sciences to determine the amount of canine blood serum containing antibodies to *E. canis* necessary to neutralize an infection in cell culture (Appendix I). The premise of the experiment was to add *E. canis* positive serum in serial dilutions to infected cell cultures to determine the minimum concentration of positive serum necessary to effectively neutralize the infection *in vitro*. After a series of methodological adaptations and improvements in aseptic techniques, the results of the trial seemed to indicate that the addition of positive serum was actually increasing the rate and degree of infection rather than neutralizing it as expected. These findings led to a series of further experiments with additional methodological adaptations that culminated in the experiments of this paper.

Experiment 1 of this paper is a final confirmation of the results from the 1999 neutralization trial showing that the addition of positive serum to infected cell cultures actually increases the rate and degree of infection of *E. canis in vitro*. Experiment 2 seeks to reproduce the results of Experiment 1 using monoclonal antibodies (Appendix II) rather than canine serum. The reason for this further adaptation to the experiment is

tied to the proposed uses of these findings. While it is difficult to say whether this *in vitro* study will have a direct correlation *in vivo*, it would certainly be useful enough merely as a culturing technique. A cell culture flask infected under traditional methods generally takes between seven and ten days to reach 100% infection. The findings of these experiments would provide a method for reaching a point of 100% infection in a shorter period of time, with the additional benefit of having an increased degree of infection (i.e. greater number of morulae per cell). However, serum containing antibodies is not a controllable or marketable substance, whereas a monoclonal antibody would be. Essentially if the same results could be demonstrated with a specifically isotyped monoclonal (or combination of monoclonals) the method could then be patented as a means of increasing the rate and degree of infection of *E. canis* in cell culture. Towards that end Experiment 2 was conducted on several different monoclonals in attempts to determine if some were more effective than others and also if this might be a site-specific phenomena.

Materials and Methods

Propagation and Maintenance of Cell Line

Ehrlichia canis is cultured in dog bone marrow cells (DBMs) due to the high concentration of leukocytes for the bacteria to infect. For this experiment cultures of DBMs suspended in a media of Fischer's media + $NaHCO_3$ + hydrocortisone + L-glutamate + 20% horse serum were obtained from the Louisiana State University School of Veterinary Medicine Cell Culture and Monoclonal lab in Corning® T-25 cell culture

flasks. These flasks and media were used throughout the experiments. Cells are obtained at the third pass (P-3) and maintained from that point until infection. In order to ensure enough nutrients and proper pH for the growth, the cells were passed every three to four days. Passage of cells involved scraping the monolayer off of the flask with a cell scraper before using a pipettor to draw the media and loose cells out of the flask. The cell suspension was then centrifuged at 800rpm for five minutes. The supernate was poured off and discarded and the pellet of cells resuspended with 1mL of fresh media per flask that cells were drawn. One-half of one milliliter of the cellular suspension was then transferred to a new T-25 flask containing 6.5mL of fresh media. In this manner the cells are passed one to two, with each original flask producing two flasks at the end of passage. Flasks were incubated at 37°C for optimal growth.

Experiment 1.- Serum Trial

Inoculation and Serum Addition

Two flasks of DBM cells P-3 were obtained and passed up until 39 flasks of DBM P-8 were obtained by the process listed above. The flasks were then broken down into three general groups with flasks 1-18 designated for positive serum, 19-36 for negative serum and 37-39 as controls with no serum added. The positive and negative serum flasks were then split into two additional groups, half designated for use with normal serum and half designated for use with serum that had been heat inactivated by heating to 80° C for 20 minutes to remove any active bacteria that could interfere with the experiment. The media in each flask was carefully drawn off without disturbing the monolayer of cells and 0.5mL of 100% *E. canis* infected DBM cells were added to each

flask. The inoculum was obtained from DBM cultures previously infected with a frozen stock culture of *E. canis*, which had been determined to be 100% infected by IFA one day prior to usage in this experiment. The flasks were then incubated for 10 minutes at 37° C to allow initial adherence of the antigen to the host cells. Next 0.5mL of Serum was added to flasks 1-36. Each flask received either untreated or heat inactivated positive or negative serum that was either undiluted or serially diluted to 1:10 or 1:100. All dilutions were made with fresh media and all serum was filtered using a Nalgene® 0.45μm syringe filter. The flasks were then incubated again for 10 minutes at 37° C before being filled to a total volume of 7mL with fresh media and incubated at 37° C.

Determination of Infection

In this experiment, infection is determined by indirect immunoflourescent antibody testing (IFA). IFA tests were performed on all 39 flasks at days seven and ten by the following procedure: A pipettor was used to make a few surface scrapes of the monolayer and then 0.5mL of cells were drawn off of each infected flask. A different pipette was used for each flask. The samples were transferred to a spin-column in a Cytospin and spun at 1000 rpm for five minutes to apply them to a slide. All slides were then fixed in a cold acetone bath for 10 minutes to ensure the cells were properly adhered to the slide. Next 50μL of filtered positive *E. canis* antibody, diluted 1:40 in non-reacted phosphate buffer serum (PBS), was applied to the cells and all slides were incubated for 30 minutes at 37°C. The slides were then washed two times in 1X PBS and once in dH_20, each wash taking five minutes on a shaker tray. Once the slides had dried, 50μ L of Rabbit-anti-dog 1:80 conjugate was applied to each slide. The slides were again incubated for 30 minutes at 37°C. Finally, the slides were washed as before and a cover

slip was applied with a drop of glycerin. The IFA results were read under a fluorescent microscope.

Experiment 2 – Monoclonal Trial

Inoculation and Monoclonal Addition

An additional two flasks of DBM cells P-3 were obtained and passed up until 30 flasks of DBM P-7 were obtained by the process listed above. The same procedure of inoculation was followed as in Experiment 1. The difference between the two experiments is solely in the addition of monoclonal antibodies rather than serum. Six distinct monoclonals (labeled A-F) to *E. canis* were obtained from the Louisiana State University School of Veterinary Medicine Cell Culture and Monoclonal Lab. Their serial numbers are listed in Appendix III. One-half of one milliliter of each monoclonal was added to each flask in place of the serum in Experiment 1, with each sample run in triplicate totaling 18 flasks for the six monoclonals. Additionally, two mixtures (A+B+D and C+E+F) were run in triplicate for a total of 24 flasks of monoclonals. A monoclonal to whole-cell *Pasteurella haemolytica* (Ph-1) was also added to three flasks to serve as a negative control, and the final three flasks had no monoclonal added (standard controls). This brought the total to 30 infected flasks. The flasks were incubated at 37°C until samples were drawn for IFA.

Determination of Infection

IFA tests were performed on days five and eight by the above listed procedure. Additionally the monoclonals were isotyped by the Louisiana State University School of Veterinary Medicine Cell Culture and Monoclonal lab using a Serotec® Mouse Monoclonal Antibody Isotyping Test Kit.

Results and Discussion

The day seven IFA results (Table 1, Figure 1) clearly demonstrate an enhanced rate of infection seen with the addition of positive serum as compared to both the negative serum and the control flasks. The heat inactivated serum did not display consistently observable results across the positive and negative serum flasks, but the flasks with heat inactivated serum did display fewer morulae per cell than those with normal serum. By the Day 10 IFA (Table2, Figure 2) the Control flasks had reached the same range of infection as the positive serum flasks in terms of percentage of infected cells while the negative serum flasks still lagged behind. However, the positive serum cells were already becoming heavily detached from the monolayer indicating that they had passed their peak log phase of growth and were starting to die off. By Day 10 it was apparent that the negative serum has some type of inhibitory effect on the infection. Additionally, there were considerably more morulae per cell in the positive serum flasks than in the control or negative serum flasks. These observations were furthered through the observation of serum addition at different dilutions. The greater the amount of positive serum added, the greater the increased rate of infection.

Table 1. Day seven IFA results of Experiment one showing undiluted, 1:10, and 1:100 dilution infection percentages for the untreated and heat inactivated positive and negative serum flasks, as well as the undiluted control flasks.

Serum	Type	Percent Infected		
		Undiluted	1:10	1:100
Positive	Untreated	80%	70%	25%
		50%	70%	25%
		80%	50%	30%
	Δ Inactivated	50%	50%	15%
		70%	30%	15%
		70%	30%	10%
Negative	Untreated	8%	7%	1%
		5%	3%	0.5%*
		5%	2%	(-)
	Δ Inactivated	15%	1%	1%
		< 1%*	1%	0.50%
		5%	0.50%	0.10%
Controls	NA	1%*	NA	NA
(No Serum)	NA	10%	NA	NA
	NA	5%	NA	NA

* indicates poor slide quality

NA = Not applicable

Slides read by Dr. Richard Corstvet, LSU Veterinary Science Department

Figure 1. Graphical presentation of Table 1 results.

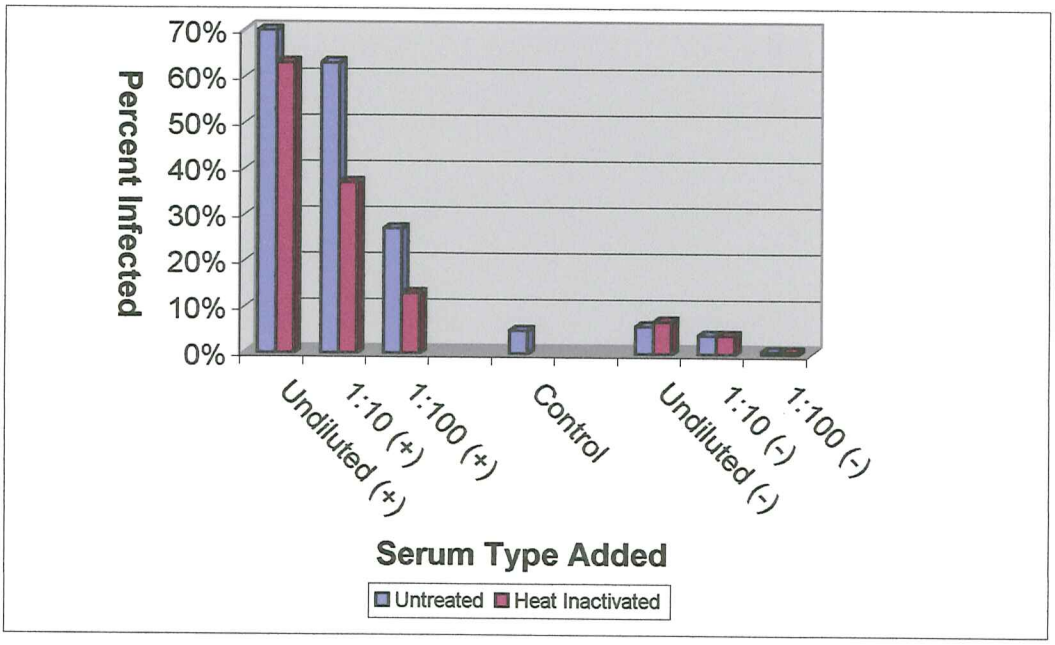
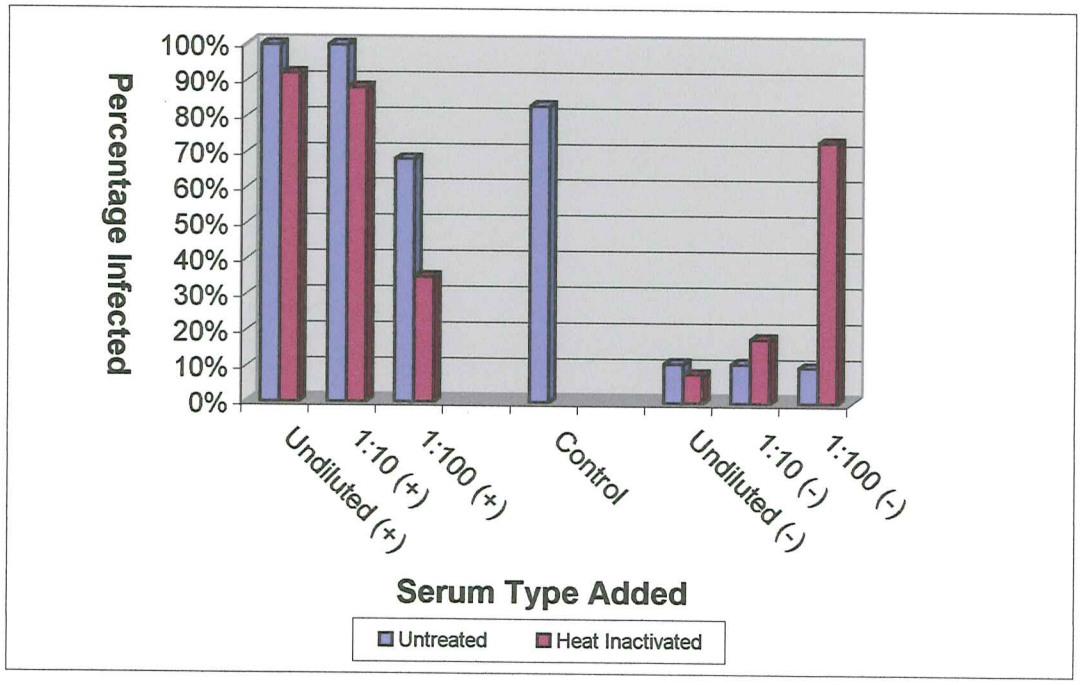


Table 2. Day ten IFA results of Experiment one showing undiluted, 1:10, and 1:100 dilution infection percentages for the untreated and heat inactivated positive and negative serum flasks, as well as the undiluted control flasks.

Serum	Type	Percent Infected		
		Undiluted	1:10	1:100
Positive	Untreated	100%	100%	80%
		100%	100%	60%
		100%	100%	65%
	Δ Inactivated	90%	65%	30%
		100%	100%	50%
		85%	100%	25%
Negative	Untreated	15%	15%	10%
		10%	8%	5%
		8%	10%	15%
	Δ Inactivated	5%	15%	50%
		15%	20%	70%
		5%	20%	100%
Controls	NA	100%	NA	NA
(No Serum)		50%	NA	NA
		100%	NA	NA

Figure 2. Graphical presentation of Table 2 results.



The IFA tests for Experiment 2 were performed earlier than those in Experiment 1 to prevent the degree of 'catch up' observed by Day 10 as the control flasks reached full infection at their normal rate. At day five (Table 3, Figure 3), all of the Ehrlichial monoclonals except C and D had reached a range of 50%-60% infection, considerable higher than the controls at an average of 15% infection. By day eight (Table 3, Figure 3) there was considerably more variation among the monoclonal infection rates. Additionally, the control flask was again able to catch up to the range of infection percentage of the other flasks. This was not surprising though since the normal *in vitro* incubation time for a flask to become fully infected is seven to ten days.

What is again not captured in these results is that all of the monoclonals (with the exception of D) still achieved a higher degree of infection than the control flasks, displaying considerably more morulae per cell. At this point, there does not appear to be a significantly determinable difference between the IgG and IgM monoclonals. If this holds true, it should indicate that the enhancement of infection is not a site-specific occurrence.

Table 3. Results of both day five and day eight IFA tests of monoclonal antibodies in Experiment 2 including the isotypes of the monoclonals.

Flask #s	Monoclonal	% Infected Day 5*	% Infected Day 8*	Isotype
1,2,3	A	57%	77%	IgG2b(k)
4,5,6	B	63%	73%	IgG2b(k)
7,8,9	C	20%	80%	IgG2b(k)
10,11,12	D	15%	67%	**
13,14,15	E	53%	60%	IgM(k)
16,17,18	F	57%	25%	IgM(k)
19,20,21	A+B+D	53%	83%	NA
22,23,24	C+E+F	56%	100%	NA
25,26,27	Ph-1 mono	Negative	40%	NA
28,29,30	Controls	15%	93%	NA

* Triplicate Means

** Titer too low to Isotype

NA = Not applicable

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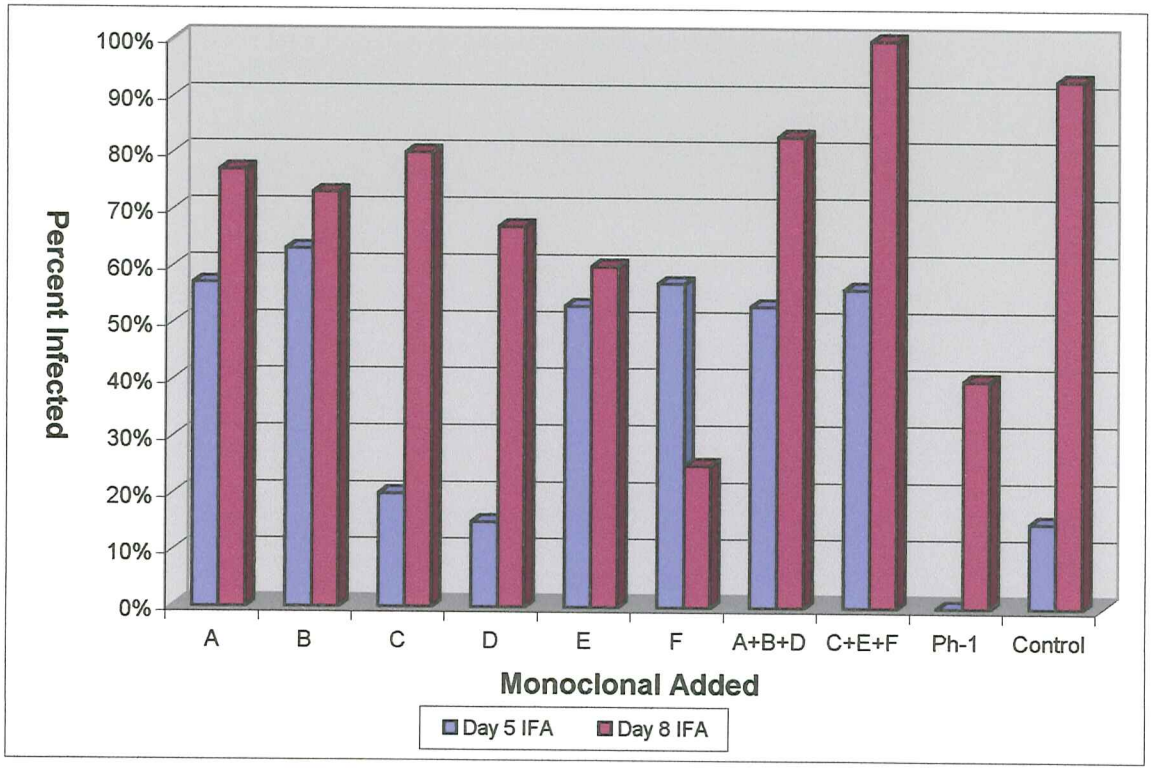
Figure 3. Graphical presentation of Table 3.



Conclusions

The results of experiments 1 and 2 do appear to verify that the addition of antibody to *in vitro* cultures of *Ehrlichia canis* actually enhances the infection rate rather than neutralizing the infection as would ordinarily be expected. While the precise reasons are not known it is hypothesized that, in accord with the normal humoral immune response, the antibody binds to the Ehrlichial cells causing increased levels of phagocytosis by the monocytes and their derivative macrophages. This process would normally neutralize infection, but since *E. canis* actually infects monocytes it indirectly causes a higher rate of infection by bringing the Ehrlichial cells into contact with their target cells more quickly.

These observations may have a variety of implications and uses. The most pertinent would be if the same effect could be observed *in vivo*. This would suggest that dogs with previous exposure to the *E. canis* antigen may be more susceptible to repeat infections owing to the stores of memory B cells in their system. However, a direct correlation to *in vivo* infection is very difficult because the dog's blood and lymphatic system would also be carrying stores of memory T cells, which should not elicit the same response as the B cell antibodies. Additionally, infection *in vivo* would not place the antigen in as dense a concentration of monocytes as occurs when DBM cultures are inoculated.

As indicated in the Introduction though, this study is primarily intended to be used in the development of a cell culture technique. With a normal infection, it takes at least seven to ten days for a flask to reach full infection. If a comparable rate and higher

degree of infection can be reached through the addition of antibody in a shorter span of time, as this study demonstrates, researchers could save increasingly valuable time in experiments involving *in vitro* infections of *E. canis*.

The primary limitation arising from this experiment was the use of IFA as a diagnostic tool. While IFA is very effective at determining infection, the reporting of results is admittedly subjective to a degree. Establishing a percentage of infected cells involves scanning a slide visually and making an estimate of the number of cells and how many of them are infected. Highly experienced researchers, such as Dr. Corstvet who read the IFA slides for these experiments, can reach very high levels of precision in their readings. However, a less-experienced slide reader may vary by ten to fifteen percentage points, establishing more of a range than a fixed numerical percentage. In order to standardize the results it is recommended that an additional test be developed to quantify the qualitative results obtained through IFA. This is also the reason that no statistics have been calculated on the results since it would be impossible to establish accurate degrees of variation and significance from qualitative data. For this experiment the IFA was acceptable because it was broad trends that were being observed between the variables at this point. However, it is impossible to fully capture, in any objective fashion, the observable increase in number of morulae per cell with the addition of antibody when recording results from IFA.

For these reasons, it is recommended that an attempt be made to quantify the results using PCR or rtPCR in conjunction with IFA as a diagnostic evaluation. The premise would be to set up a similar experiment to those in this paper and scrape samples for PCR from each flask at several days within the first week of infection. DNA would

be extracted from each sample and serially diluted. PCR would then be performed on each dilution. If the flasks with antibody added did indeed contain greater amounts of antigen, then the antigen should be present at further dilutions than those flasks without the addition of antibody, which would be easily observable by running PCR on all dilutions.

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Appendix I

1999 *E. Canis* Experiment – Serum Neutralization

Introduction

This is a summary report of the initial experimentation begun in the spring of 1999 that led to the developments of this thesis. The intention was to formulate a method for neutralization of *Ehrlichia canis* in infected dog bone marrow cells (DBMs) through exposure to +*E. canis* dog serum. What follows are the procedures that were developed and adapted and the results from these initial experiments.

Materials and Methods

The DBMs are usually cultured in Corning® T-25 cell culture flasks in a media of Fisher's + $NaHCO_3$ + 20% Horse Serum + L-glut + Hydrocortisone. One-half of one milliliter of cells would be added to 7mL of media. Intending to run several dilutions of the serum trial, an alternate method was sought for raising the DBMs in a matter more conducive to a greater number of samples. It was decided to attempt to culture the DBMs in multi-well cell culture plates. The Costar® 6 well plates were chosen over the smaller 16 well plates. It was felt that the increased surface area for the *E. canis* monolayer in the 6 well plates would be closer to the growing environment in the T-25 flasks.

The Costar® plates were compared proportionally with the T-25 flasks and the volumes of media and cells were initially halved to 3.5mL of media and 0.25mL of cells per well since the wells held approximately half the total volume of the T-25 flasks. An

initial trial was run on two plates. One was sealed with parafilm to attempt to reproduce the airtight environment of the flask and the other was covered but not sealed. After three days the cells in the sealed plate had a few more floating cells than normal, but were confluent and generally healthy. The cells in the unsealed plate had many floating clumps, were less confluent and appeared to have a poor pH. It was determined that the culture plates would be satisfactory to use so long as they were kept well sealed.

The sealed plate was then infected with 0.25mL per well of *E. canis* infected DBM P-28 (IFA=100% infected) to ensure that the culture plates would also ensure the proper environment for infection of the DBMs. The inoculum was diluted serially from undiluted, 1:10, 1:100, 1:1,000, 1:10,000, 1:100,000 for the six wells. Before the inoculum was added, the old media was drawn off and replaced with 3.5mL of fresh media per well. The inoculum was then added to each well and the plates were resealed with parafilm and incubated. IFA tests were performed on a sample of each dilution and on the plate itself after five days. (Table A-1) In general, the infected cells in the plate looked slightly more beat up than they normally would in a T-25 and contamination was suspected. It was decided that the plates would also be satisfactory for the infection of the DBMs, but it was also recognized that meticulous aseptic technique would be crucial.

Table A-1. Results of IFA used to test the usability of the Costar® six-well plates showing the percentage of infection in original samples and five days post infection at six serial dilutions.

Well #	Inoculum Dilution	% Infection Original Sample	% Infection Day 5
1	Undiluted	100%	70%
2	1:10	less cells, 100%	50%
3	1:100	still less cells, 100%	30%
4	1:1000	approx. 5 cells, 100%	(-)
5	1:10000	no infected cells	(-)
6	1:100000	1 infected cell, 100%	(-)

Serum Trial 1

In attempting to neutralize the *E. canis,* dog serum was added to the infected DBMs. Presumably, the addition of +*E. canis* serum would neutralize the *E. canis* whereas the addition of –*E. canis* serum would not. In every trial a third group was tested using 1X PBS to form a control group. In the first trial, the inoculum was diluted 1:100, and the serum was diluted for the six wells 1:5, 1:10, 1:20, 1:40, 1:80, 1:160. The trial was run in two different sets, involving two different methods. Each set had a +, –, and control plate making a total of six plates (36 wells). The plates used had been started with DBM P29 three days earlier, the inoculum used was an 80% infected +*E. canis* DBM P26.

In Set 1 the old media was first drawn off of each well of three culture plates. To the first plate 0.25mL +*E. canis* serum was added directly onto the monolayer. To the second plate 0.25mL –*E. canis* serum was added, and to the third plate sterile 1X PBS was added. The plates were incubated for ten minutes at 37°C and 5%CO_2. After ten minutes, 0.25mL of the inoculum was added and the plates were again incubated for ten minutes. Finally, the plates were refilled with 3.5mL of fresh media, sealed with parafilm and replaced in the incubators.

Set 2 was prepared in the same manner except the inoculum and serum/control were first combined together in a 5mL round bottom tube and incubated together for ten minutes rather than adding each individually to the monolayer. After they were incubated together, they were added to the monolayer and incubated for another ten minutes before the media was replaced and the plates were sealed and incubated. IFA tests were run on all 36 wells on Days 4 and 7 and on selected wells on Day 15 (A-2).

Table A-2. IFA results for Trial 1 at days 4, 8, and 14 showing the first attempt at the actual serum neutralization trial that was conducted in the Costar® six-well culture plates on positive and negative serum as well as controls at six dilutions each using two different methods to combine the serum and antigen.

Slide number	Set number	Plate type	Serum Dilution	Day 4 IFA*	Day 8 IFA**	Day 14 IFA***
1	1	(+)	1:05	(-)	0.10%	1%
2	1	(+)	1:10	(-)	0.20%	(-)
3	1	(+)	1:20	(-)	0.30%	(-)
4	1	(+)	1:40	(-)	0.10%	(-)
5	1	(+)	1:80	(-)	0.10%	(-)
6	1	(+)	1:160	(-)	0.10%	(-)
7	1	(-)	1:05	(-)	(-)	No IFA
8	1	(-)	1:10	(-)	(-)	No IFA
9	1	(-)	1:20	(-)	(-)	No IFA
10	1	(-)	1:40	(-)	(-)	No IFA
11	1	(-)	1:80	(-)	(-)	No IFA
12	1	(-)	1:160	(-)	(-)	(-)
13	1	control	NA	(-)	1%	No IFA
14	1	control	NA	(-)	(-)	No IFA
15	1	control	NA	(-)	0.50%	No IFA
16	1	control	NA	(-)	(-)	No IFA
17	1	control	NA	(-)	0.10%	No IFA
18	1	control	NA	(-)	0.50%	(-)
19	2	(+)	1:05	(-)	3%	No IFA
20	2	(+)	1:10	(-)	(-)	No IFA
21	2	(+)	1:20	(-)	0.10%	No IFA
22	2	(+)	1:40	(-)	0.10%	No IFA
23	2	(+)	1:80	(-)	(-)	No IFA
24	2	(+)	1:160	(-)	0.10%	(-)
25	2	(-)	1:05	(-)	(-)	No IFA
26	2	(-)	1:10	(-)	(-)	No IFA
27	2	(-)	1:20	(-)	(-)	No IFA
28	2	(-)	1:40	(-)	0.10%	No IFA
29	2	(-)	1:80	(-)	(-)	No IFA
30	2	(-)	1:160	(-)	0.10%	(-)
31	2	control	NA	(-)	0.50%	No IFA
32	2	control	NA	(-)	0.50%	No IFA
33	2	control	NA	(-)	0.50%	No IFA
34	2	control	NA	(-)	0.10%	No IFA
35	2	control	NA	(-)	0.10%	No IFA
36	2	control	NA	(-)	0.10%	(-)

*IFA performed on 3/5/99
**IFA performed on 3/8/99
***IFA performed on 3/15/99
NA = not applicable

Trial 2

As a result of the problems incurred in Trial 1 in attempting to manage such a large number of samples and the problematic nature of the culture plates in regards to the possibility of contamination, the remaining trials were conducted in T-25 culture flasks and less dilutions were used. Trial 2 was conducted on undiluted serum only. Three T-25 flasks were prepared following the same general procedure as found in Trial 1-Set 1 above. The flasks had already been prepared with DBM P-35. The media was first drawn off. The serum or PBS was then added (0.5mL) and the flasks were incubated for ten minutes. Next the inoculum, undiluted +*E. canis* infected DBM P-35, was added and the flasks were incubated again. Finally the media was replaced and the flasks were returned to the incubators. IFA testing was performed on the flasks after 13 days. The control flask was 20% infected. The two serum flasks were not infected at all, but appeared contaminated by some other bacteria that may have been naturally present in the serum.

Trial 3

Trial 3 was conducted in the same method as Trial 2, except the serum was filtered with a Nalgene® 0.45μm syringe filter. Three flasks of DBM P22 were infected with 80% *E. canis* infected DBM P21. IFA tests were run after seven days. The +serum flask was 100% infected, the –serum flask was 10% infected, and the control flask was 50% infected.

Trial 4

A fourth trial was conducted to confirm the results of Trial 3. The same procedure from Trial 2 was followed, with the addition of the filtered serum from Trial 3. An additional 1:10 dilution was run on the + and – serum. Five flasks of DBM P24 were infected with 50% *E. canis* infected DBM P22. IFA tests were conducted on Days 7 and 10. The results are listed in Table 3.

Table A-3. Results of the days seven and ten IFA tests of Trial 4 using only undiluted and 1:10 diluted positive and negative serum against the control flask.

Slide number	Serum type	Serum dilution	Day 7 IFA	Day 10 IFA
1	(+)	undiluted	10%	40%
2	(+)	1:10	5%	20%
3	(-)	undiluted	(-)	(-)
4	(-)	1:10	0.50%	(-)
5	control	NA	3%	0.50%

NA = not applicable

Discussion

Once a working method for the neutralization trials was developed (Trials 3 and 4), the results were not in agreement with the initial hypothesis. Rather than neutralizing the *E. canis*, the + serum actually caused a greater amount of infection than the control. The – serum on the other hand, which presumably would not have affected the infection rate, appears to have lowered the amount of the infection below that of the control. No speculations have been made thus far as to the reasons for these results and the results themselves are fairly inconclusive due to the small number of actual trials. Repeated trials would have to be performed to get a more definite confirmation of these results before any conclusion can be reached.

Appendix II

Discussion of Monoclonal Antibodies

Antibodies found in blood serum are polyvalent, containing a variety of B-cell clones arising from different epitomes on the antigen. Monoclonal antibodies are derived from a single plasma cell and are specific for a single epitome of a complex antigen. Monoclonals are produced by merging B-cells from a mouse injected with a specific antigen (*E. canis* in this case) with myolomas (A-cells), cancerous tumors of plasma cells. The merged cells (hybridomas) are then separated from any parent A or B cells. The initial hybridoma mixture may include residual homogenous A-A or B-B cells instead of the desired A-B hybrid. A selective medium called HAT (hypoxanthine, aminopterine, and thymidine) is used to select for the A-B hybridomas. The principle behind the hybridoma is the combination of the antibody-producing B cell with the tumor cells' rapid growth and division properties. The hybridomas are then cultured in separate wells and tested periodically for antibody production. If more than one type of antibody is detected the wells are split and retested until each well contains hybridomas producing only one type (monoclonal) of antibody. The clones are then propagated either in cell culture or in the ascites fluid of a mouse to increase their volume.[5]

Appendix III

Monoclonal serial numbers*

A: 6-40-8-30-77

B: 6-40-25-4

C: 6-40-8-29-69

D: 6-24-6-28

E: 6-24-3-5

F: 6-25-40

* Monoclonals obtained from stock at the Louisiana State University School of Veterinary Medicine Cell Culture and Monoclonal Lab, Baton Rouge, LA