PN-ABM-643

Washington State University

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July 11, 1991

Agency for International Development
PPC/CDIE Room 215 SA-18
Washington DC 20523

RE: DPE-5542-G-SS-7008-00

Dear Sir or Madam:

I have enclosed a copy of the progress report for the above referenced grant, "Development of a synthetic peptide vaccine against bovine anaplasmosis". The progress report covers the period ending June 30, 1991. This project has been granted a no-cost 1-year extension until December 31, 1991. The project has met the objectives as described in the current report. In addition, our most recent NRC review has been highly favorable. Please contact me if I can be of any assistance or provide you with additional information regarding this project.

Sincerely,

Guy H. Palmer DVM, PhD

PN-ABM-643
75758

PROGRESS REPORT: GRANT No. DPE-5542-G-SS-7008-00

Development of a peptide vaccine for protection against bovine anaplasmosis

Subgrant PI: Guy H. Palmer
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Reporting Period: 1 January 1991 through 30 June 1991.

Goals of the Project: The failure to develop effective immunoprophylaxis against anaplasmosis results from the complexity of the disease, including persistence of the parasite and from lack of application of current research technology to the disease.

We have cloned the gene for *Anaplasma marginale* MSP-1 (AmF 105) with the objective of deriving the primary structure of the native polypeptide. Determination of the structure of MSP-1, specifically the protection-inducing epitope, will allow development of synthetic peptide analogs for immunization of cattle. Synthetic peptide immunogens have several unique properties which may be critical to effective use of a subunit vaccine: [1] the protection-inducing peptide can be efficiently synthesized in vitro without the requirement for large scale fermentation followed by purification of the single expressed protein from *E. coli* host cells; [2] the presentation of the key protection-inducing epitopes, especially if linked in tandem repeats, may focus the immune-response more effectively on this isolate-common epitope than vaccines containing extraneous proteins; [3] the peptide based vaccine carries no risk of reversion to virulence or as a threat to immunodeficient animal caretakers. The recent success of synthetic peptides as a protective immunogen in malaria clearly demonstrates the feasibility of the molecular biology - synthetic epitope approach. The identification of MSP-1 as bearing an invariant peptide epitope capable of inducing protection in immunized cattle, combined with recent advances in molecular biology, make development of an effective synthetic peptide vaccine an achievable goal. The research will also provide needed insight into genomic-antigen relationships in rickettsial pathogens and has the potential to demonstrate the effectiveness of a molecular biological approach to solving animal disease problems.

The specific aims to attain these goals are:

1. Sequence the gene coding for MSP-1 (AmF 105) and derive the primary structure of MSP-1.

2. Synthesize peptide analogs of the MSP-1 protection-inducing epitope based upon primary structure of MSP-1.

3. Demonstrate the effectiveness of a synthetic peptide analog of the protection-inducing MSP-1 epitope as a protective immunogen.

Progress to date: Reported project progress has established a basis for the testing of a synthetic peptide polymer as a protective immunogen against disease caused by acute *A. marginale* infection. This research was recently published in the Proceedings of the National Academy of Sciences, USA[1] and is briefly summarized below.

1. localization of the neutralization-sensitive epitope on MSP1a (AmF105U) polypeptide to the tandemly repeated sequence: S-A-G-G-Q-Q-Q-E-S-S-V-S-S-Q-S-D(EA)-Q-A-S-T-S-S-Q-L-G-A-D-S-S.
2. identification of the precise monoclonal binding site within this sequence to a six amino acid peptide, Q(E)ASTSS.
3. demonstration that this sequence, originally identified in the Florida isolate, is completely conserved among three additional U.S. isolates, Virginia, South Idaho, and North Texas. Research on the Zimbabwe isolate is detailed below.

In initial immunization trials in cattle, (collaboration with Dr. Anthony Barbet of the University of Florida) carbodiimide polymerized D-S-S-S-A-G-G-Q-Q-Q-E-S-S-V-S-S-Q-S-D-Q-A-S-T-S-S-Q-L-G-A peptide induced significant immune responses in 9/10 vaccinates (reported for the period ending June 30, 1990. We have completed the synthetic peptide immunization and challenge. These results were reported for the period ending December 31, 1990. Three groups of cattle were immunized: Group 1 (synthetic peptide); Group 2 (native MSP-1); Group 3 (control). Groups 1 and 2 developed significant antibody titers and lymphocyte responses (see Table 1) while control animals were unresponsive. Upon challenge, 4/5 Group 1 synthetic peptide immunized cattle were protected and 5/5 native MSP-1 immunized cattle were protected as assessed using peak rickettsemia responses compared to control immunized cattle.

Group	Peak Rickettsemia	Ab Titer[1]	Ab Titer[2]	LSI[3]
Peptide[4]	1.8	10^3	10^4	7.1
MSP-1	1.4	10^3	10^3	16.2
Control[5]	5.0	0	0	1.0

[1]Ab titer to polymerized 29mer peptide
[2]Ab titer to native MSP-1
[3]Lymphocyte Stimulation Index vs. MSP-1
[4]n=4, the fifth animal was not protected.
[5]Immunized with adjuvant only on an identical schedule

As noted in our previous progress reports, in Zimbabwe we have identified and isolated a local field strain of *Anaplasma marginale* for molecular characterization and challenge of vaccinates. The isolation and characterization of the this strain was completed solely at the Veterinary Research Laboratory based on collaborative project design occurring during Dr. Palmer's 1988 research visit in Zimbabwe. This strain, designated the Norton, Zimbabwe strain, is currently maintained in the Veterinary Research Laboratory. Dr. Ntando Tebele, Research Officer at the

Veterinary Research Laboratory, completed an initial animal trial in which cattle infected with the prototype Florida isolate (vaccine work to date is based on this isolate as it has been shown to be the most widely cross-protective of all isolates tested) were challenged with either the Florida isolate or the recent Zimbabwe isolate. Significantly, cattle infected with Florida *A. marginale* were protected against homologous challenge but only partially protected against heterologous Zimbabwe isolate. This finding is dissimilar to the protection seen with heterologous Virginia challenge of Florida infected cattle. This may reflect a divergence in protection-inducing epitopes between the Florida isolate and the Zimbabwe isolate. The manuscript describing the isolation and characterization of the cross-protective immunity is currently in press for publication during 1991[2].

We hypothesize that the variation in protection is due to strain variation in surface exposed membrane epitopes. Previously we have shown epitope variation among different strains of *A. marginale*[3] and recently, documented cyclic rickettsemia in persistently infected cattle, an observation consistent with intrastrain antigenic variation[4]. Dr. Tebele has recently completed characterization of the membrane polypeptides and exposed epitopes of the Norton, Zimbabwe strain of *A. marginale*. This research confirmed conservation of epitopes on *A. marginale* membrane polypeptides MSP-1a (including the six residue neutralization-sensitive epitope on a 65kD polypeptide), MSP-1b, MSP-2, and MSP-3 (Table 2).

TABLE 2. Conserved surface polypeptide epitopes between the Florida and Norton Zimbabwe strains of *A. marginale*

Monoclonal antibody	Polypeptide bound[a]	Strain Reactivity	
		U.S. strains	Norton Zimbabwe strain
Ana 22B1	MSP-1a	9/9[b]	+
AmR R38A6	MSP-1b	1/1[c]	-
Ana F19E2	MSP-2	9/9[b]	+
43/19.3.5	MSP-3	1/1[c]	-
43/23.2.8	MSP-3	1/1[c]	+
Ana F16C1	AmF 19	1/1[c]	+
Tryp 1E1[d]	*T. brucei*	0/9[b]	-

[a] See ref.11,12,22,23.
[b] Strains used are Florida, Idaho, Kansas, Missouri, North Texas, Oklahoma, Virginia, Washington-C, and Washington-O (22).
[c] Only the Florida strain tested to date.
[d] Monoclonal antibody to a *Trypanosoma brucei* variable surface glycoprotein used as a negative control.

In addition, several membrane antigens were described for the first time using the Norton, Zimbabwe strain including 55, 31, 30, 28, 26.5, and 25 kD antigens. Immunization of cattle with membrane preparation containing this peptide solidly protected cattle against the Norton, Zimbabwe strain (Table 3). All outer membrane immunized calves were protected from clinical disease as demonstrated by significantly lower decreases in PCV and Hb when compared to non-immunized and saponin immunized calves. Decreases in PCV and Hb are a measure of the severity of anemia which is a prominent clinical sign of anaplasmosis. Significantly, all immunized calves with antibody titers $\geq 10^4$ against outer membrane polypeptides were completely protected from microscopically detectable rickettsemia.

TABLE 3. Correlation of antibody titers and protection in *A. marginale* outer membrane immunized calves

Animal number	Antibody titer[a]	Peak rickettsemia (%)	Decrease in PCV (%)[b]	Decrease in Hb (%)[c]
3	10^6	0	20.6	21.6
15	10^6	0	17.6	19.8
7	10^5	0	22.6	25.8
11	10^5	0	21.9	22.6
22	10^3	2.3	33.3	35.6

[a] Pre-challenge antibody titers against Norton Zimbabwe strain initial bodies.
[b] Increase in antibody titer has a negative linear relationship with change in PCV ($r = -0.97$; $r^2 = 0.95$, $P < 0.05$).
[c] Increase in antibody titer has a negative linear relationship with change in Hb ($r = -0.96$; $r^2 = 0.94$, $P < 0.05$).

Diluted sera from calves immunized with outer membranes recognized six polypeptides, two of which share epitopes with surface exposed polypeptides of the Florida strain. A 31 kD polypeptide was common to two membrane preparations that induced protection. Protection in membrane immunized calves could be due to responses to antigens unique to the Norton Zimbabwe strain, antigens conserved among strains (such as MSP-2 and AmF19), or a combination of unique and common antigens. Further fractionation of membranes and immunization trials will help identify those Norton Zimbabwe strain polypeptides which are critical to inducing protective immunity.

This research demonstrated a significant relationship between increase in antibody titer and protection against anemia. Coefficient of determination values indicate that 81% - 95% of protection from anemia can be predicted by antibody titer (18). All calves with antibody titers greater than 10^3 against membrane antigens were completely protected from rickettsemia. Based on this correlation, we plan to determine the effector role of antibody using immunoglobulin transfer from membrane immunized calves.

These results will be published in the September issue of **Infection and Immunity**.

TABLE 4. Responses of immunized and non-immunized calves to challenge with the Zimbabwe strain of *A. marginale*

Immunogen	Mean[a] decrease in PCV (%)	Mean[a] decrease in Hb (%)	Peak rickettsemia (%)
Fraction 1 immunized	29.7±16.4*	32.3±16.5*	1.3 ± 2.0[b]
Fraction 2 immunized	23.2±5.9*	25.12±6.3*	0.5 ± 1.0[c]
Saponin control	52.0±4.9	54.7±4.9	11.8±4.6
Non-immunized	49.8±6.9	51.2±6.4	12.1±5.5

[a] Mean ±SD.
[b] Only 2 out of 5 calves developed rickettsemias.
[c] Only 1 out of 5 calves developed a rickettsemia.
* All means with an asterisk were significantly different from saponin control and non-immunized calves.

Collaboration with Zimbabwe: The focus of the collaboration and strengthening the research capacity at the Veterinary Research Laboratory has been the research training of Dr. Ntando Tebele. Dr. Tebele's training is summarized below:

1. She has completed her M.S. with a focus on microbial immunology/molecular biology at Washington State University in Spring 1991. [grade point average 4.0/4.0; 13 of 19 graded graduate course semester hours focused either directly on molecular biological techniques (Micro 462, Micro 590) or on the research application of molecular techniques to solving problems in animal disease (VPa 544, VPa 545, VPa 548).
2. All M.S. research was done in Zimbabwe where she has a permanent appointment as a Veterinary Research Officer in the Veterinary Research Laboratory. This program emphasizes on site research in order to facilitate technology transfer and to encourage independent development.
3. She has written two first author publications resulting from her project research done 100% at the Veterinary Research Laboratory.

   Tebele, N. and Palmer, G.H.: Crossprotective immunity between the Florida and a Zimbabwe isolate of *Anaplasma marginale*. Tropical Animal Health and Production. In press (1991).

Tebele, N., McGuire, T.C. and Palmer, G.H.: Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infection and Immunity, In press (Sept 1991).

4. To broaden her exposure to research in the U.S., Dr. Tebele attended the Eighth National Veterinary Hemoparasite Disease Conference in St. Louis during April 11-12, 1989. In addition, she attended the advanced program (USAID/National Research Council, Sept. 1990) on use of the polymerase chain reaction in biotechnology research in lesser developed countries.
5. In designing her program, we included a course on research proposal preparation (EdAd596 Grant Proposals) with the goal of facilitating her development as an independent scientist competitive for research funding independent of a U.S. collaborator.

Publications resulting from project:

1. Tebele, N. and Palmer, G.H.: Crossprotective immunity between the Florida and a Zimbabwe isolate of *Anaplasma marginale*. Trop. Animal Health and Prod. In press.
2. Tebele, N., McGuire, T.C. and Palmer, G.H.: Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infection and Immunity, In press (Sept 1991).
3. Allred, D.R., McGuire, T.C., Palmer, G.H., Leib, S.R., Harkins, T.M., McElwain, T.F., and Barbet, A.F.: Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc. Natl. Acad. Sci. USA, 87:3220-3224, 1990.
4. Palmer, G.H., Barbet, A.F., Cantor, G.H., McGuire, T.C.: Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. Infect. Immun. 57:3666-3669, 1989.
5. Oberle, S.M., Palmer, G.H., Barbet, A.F. and McGuire, T.C.: Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. Infect. Immun. 56:1567-1573, 1988.
6. Palmer, G.H.: Anaplasma Vaccines. In Veterinary Protozoan and Hemoparasite Vaccines, Wright, I.G., Ed. pp. 1-29. CRC Press, Boca Raton. 1989.

Conference proceedings resulting from project:

1. Palmer, G.H., Barbet, A.F., Davis, W.C., Oberle, S.M., McGuire, T.C.: Characterization of *Anaplasma marginale* surface protein MSP-2 (36 kD) as a protective immunogen. In Proceedings of the Eighth National Veterinary Hemoparasite Disease Conference, R.J. Hidalgo, Ed. pp. 491-498. Louisiana State University, 1989.
2. Shkap, V., Pipano, E., Bin, H., Barbet, A.F., Davis, W.C., McGuire, T.C., Palmer, G.H.: Conservation of surface protein epitopes between *Anaplasma centrale* and *Anaplasma marginale*: a review of the basis for vaccine development. In Proceedings of the Eighth National Veterinary Hemoparasite Disease Conference, R.J. Hidalgo, Ed. pp. 499-506. Louisiana State University, 1989.In Press.

3. McGuire, T.C., Palmer, G.H., Allred, D.R., Davis, W.C., Barbet, A.F.: Characterization of an immunoprotective surface protein complex of *Anaplasma marginale* by DNA cloning and expression. In Proceedings of the Eighth National Veterinary Hemoparasite Disease Conference, R.J. Hidalgo, Ed. pp. 465-476. Louisiana State University, 1989.

International network communications resulting from project:

1. Harrington, R.D., Palmer, G.H., and Knowles, D.P.: *Anaplasma* and *Babesia* vaccines. In Anaplasmosis and Babesiosis Network Newsletter, Volume 1, No. 3, pp.4-5. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.
2. Kieser, S.T., Knowles, D.P., and Palmer, G.H.: Virus vectors: a new era in vaccine technology. In Anaplasmosis and Babesiosis Network Newsletter, Volume 1, No. 4, pp.2-3. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.
3. Kieser, S.T., Palmer, G.H., and Knowles, D.P.: Mechanisms of immunity in hemoparasitic diseases. In Anaplasmosis and Babesiosis Network Newsletter, Volume 2, No. 1, pp.3-4. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.
4. Trueblood, E.S. and Palmer, G.H.: Anaplasmosis: a review of diagnostic techniques. In Anaplasmosis and Babesiosis Network Newsletter, Volume 2, No. 3, pp.2-4. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.
5. Raymond, R.J., Palmer, G.H., and Knowles, D.P.: The polymerase chain reaction: a new tool for detecting hemoparasitic diseases. In Anaplasmosis and Babesiosis Network Newsletter, Volume 2, No. 4, pp.1-2. Published by the Washington State University International Project Support Office with funding by USAID DAN-4178-A-00-7056-00.

Literature Cited:

[1]Allred, D.R., McGuire, T.C., Palmer, G.H., Leib, S.R., Harkins, T.M., McElwain, T.F., and Barbet, A.F : Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc. Natl. Acad. Sci. USA, 87:3220-3224, 1990.

[2]Tebele, N. and Palmer, G.H.: Crossprotective immunity between the Florida and a Zimbabwe isolate of *Anaplasma marginale*. In press.

[3]McGuire, T.C., Palmer, G.H., Goff, W.L., Johnson, M.I. and Davis, W.C.: Common and isolate restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. Infect. Immun. 45:697-700, 1984.

[4]Kieser, S.T., Eriks, I.S., and Palmer, G.H.: Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. Infect. Immun. 58:1117-1119, 1990.

[5]Tebele, N., McGuire, T.C. and Palmer, G.H.: Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infection and Immunity, In press (Sept 1991).