Purification, biotinylation, and testing of a monoclonal antibody to identify B cells in *Trachemys scripta*, the red-eared slider turtle.

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ABSTRACT

There is a shortage of research in reptile immunity which is further hampered by lack of reptile-specific reagents. Evidence suggests there are important differences between reptile and mammalian immune strategies and our laboratory is interested in reptile B cell development and function. Our undergraduate research project involved the preparation of a previously developed monoclonal antibody (HL673) that recognizes turtle light chain protein. To begin, culture supernatant from the HL673 mAb murine cell line was received and was applied to a protein A affinity column. Unbound proteins were then washed away, and the bound proteins were removed from the column using low pH glycine-HCl buffer. The purified antibody proteins were collected in fractions, OD at 280nm measured, then positive fractions were pooled and dialyzed. The concentration of the purified antibodies was determined, and reactivity tested using an ELISA plate coated with dilute turtle serum. Dilutions of the purified HL673 were detected by anti-mouse IgG-horseradish peroxidase (HRP). Furthermore, some of the antibody preparation was conjugated to biotin. After dialysis, the HL673-biotin was tested by ELISA with dilute turtle serum and detected by streptavidin-HRP. The newly biotinylated antibody was incubated with blood and spleen samples from both adult and hatchling red-eared slider turtles. Bound antibodies were detected using streptavidin-fluorochrome and B cell populations identified using flow cytometry. Our results showed successful detection of turtle B cells using the labeled mAb in both hatchling and adult turtle cell samples. Future studies will use this reagent to investigate the distribution and function of B cells in reptile gut immunity. This work was supported by NSF 1725199 and NIH 1R15AI140118 – 01.

BACKGROUND

- Reptiles are vertebrate ectotherms that have both innate and adaptive immunity like people.
- While they are known to possess primary lymphoid tissues such as bone marrow and a thymus, reptiles lack certain secondary lymphoid tissues such as lymph nodes.
- Turtles have long lifespans and live in pathogen-rich environments, therefore it is very likely that they have robust immunity, but we know very little about their immune system.
- Turtles have B cells that can produce antibodies.
- B cell identification is important to study turtle immune responses.

METHODS

- A monoclonal antibody (called HL-673) that binds to turtle antibody proteins was purchased from the University of Florida.
- We used the protein A affinity column purification method to purify the HL-673 protein away from other proteins in the sample.
- We tested the reactivity of the HL-673 by ELISA after purification. Some of the HL-673 protein was chemically coupled to biotin for use in other experiments.
- A wild population of red-eared sliders were captured in the field and brought back to the lab for study.
- Spleen samples were collected from some adults and hatchlings (>6 weeks).
- White blood cells were purified using centrifugation over a density gradient.
- Cells were counted with trypan blue, stained with HL-673-Biotin, and analyzed via flow cytometry on FACSMelody.



Photo credits: Laura Zimmerman and Marc Ashford

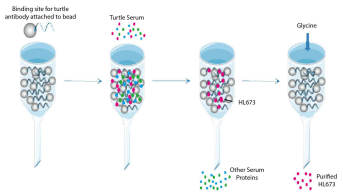
ISOLATION OF HL673 MAB USING AFFINITY CHROMATOGRAPHY.

Hypothesis: HL673 can be isolated from other proteins in the purchased culture supernatant using affinity chromatography.

Prediction: The HL673 will bind to the protein A on the column until Glycine buffer removes it.

Experiment: Tubes of purchased supernatant were thawed and the column was washed with saline until the liquid coming out (flow through) had an OD 280nm of less than 0.05. Supernatant was run through the column and it was washed with saline until the OD was low. We then added Glycine buffer (pH 2.5) to remove HL673 that was bound. Collection tubes were filled with 500 ul of Tris buffer (pH 8.) to neutralize the acid. We collected approximately 500 uL of the flow through in each tube. The OD for each tube was tested. We continued collecting until the A280 value was less than 0.05, which indicated that all HL673 was removed from the column.

Results: The HL673 was successfully isolated.



Batch #1 OD 280nm	
1) 0.092	
2) 2.494	Pooled
3) 1.139	
4) 0.090	
5) -0.008	
6) -0.021	
Batch #2 OD 280nm	
1) 0.045	
2) 0.077	
3) 0.138	
4) 2.028	Pooled
5) 3.229	
6) 0.985	
7) 0.026	
8) 0.008	

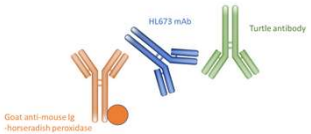
PURIFIED FRACTIONS CONTAIN MOUSE ANTIBODY PROTEINS AND BIND TURTLE ANTIBODIES

Hypothesis: The HL673 will bind to antibodies in turtle serum when tested by ELISA.

Prediction: The ELISA wells with more HL673 will have a darker color if the antibodies have bound to the turtle serum on the plate.

Experiment: The ELISA plate was coated with turtle serum diluted 1:10 in 1x PBS. After blocking the plate, samples were added to each well. Buffer was added as a negative control, a positive control protein was also added along with our test sample. These samples were diluted two-fold down each respective column. Following incubation and washing, goat anti-mouse IgG-HRP was added for detection of the HL673. After incubation, and washing substrate was added. The plate was read at 405 nm.

Results: The purified HL673 showed successful binding to the turtle serum.



Dilution	- Control	HL673	+ Control
1:20	0.241	0.651	0.659
1:40	0.234	0.669	0.649
1:80	0.212	0.637	0.598
1:160	0.223	0.656	0.64
1:320	0.228	0.673	0.654
1:640	0.234	0.653	0.652
1:1280	0.223	0.62	0.622
1:2560	0.236	0.598	0.582

HL673 MAB CONJUGATED TO BIOTIN IS ACTIVE

Hypothesis: The HL673 coupled to the biotin during the coupling procedure.

Prediction: The ELISA wells will be darker if the HL673 is biotinylated, because the Streptavidin-HRP will bind the biotin, and produce color with the substrate.

Experiment: An ELISA plate was coated with diluted turtle serum in PBS. The wells were washed, and samples were added. Buffer was used as the negative control, a previously biotinylated protein was used as a positive control, and our test sample was added. Then serial two-fold dilutions were performed. The plate was incubated and washed. Streptavidin-HRP was diluted 1:1000 and incubated followed by washing. Substrate for the HRP was added to each well, which allowed color change correlating to the amount of HL673 bound.

Results: The biotin was coupled successfully.

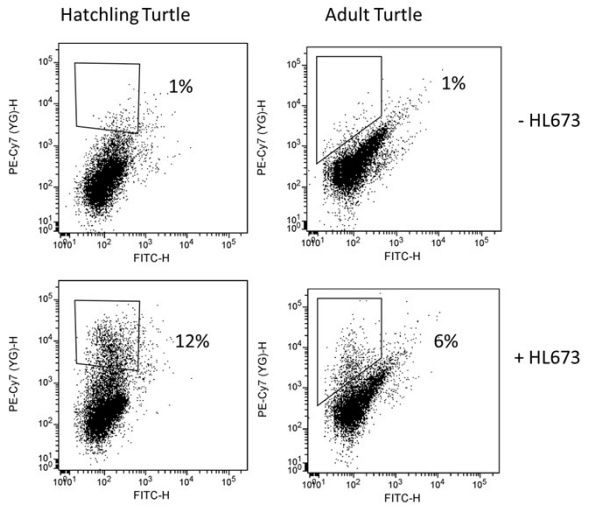
Dilution	- Control	+ Control	Biotinylated HL673
1:20	0.197	0.3	0.53
1:40	0.234	0.335	0.62
1:80	0.221	0.331	0.461
1:160	0.213	0.433	0.387
1:320	0.149	0.224	0.218
1:640	0.132	0.186	0.185
1:1280	0.152	0.179	0.173
1:2560	0.137	0.215	0.145

HL673-BIOTIN CAN DETECT B CELLS IN SPLEENS OF HATCHLINGS AND ADULTS

Hypothesis: HL673-biotin will bind to surface Ig on turtle B cells

Prediction: Using flow cytometry, we will be able to detect B cells in the spleens of hatchlings and adults after staining with HL673 biotin.

Experiment: Spleens were collected and pooled from 8 individual hatchling turtles or individual adult turtles. Single cell suspensions were prepared in Ringer's solution. White blood cells were isolated by percoll gradients, counted and stained with HL673-biotin mAb. Bound antibodies were detected with streptavidin-PECy7 and analyzed by FACSMelody. Representative dot plots are shown gated on live cells. Boxed regions indicate percentage of B cells. Amount of HL673 binding is shown on Y axis (PE-CY7). Top plots: samples without HL673 added. Bottom plots: with HL673 added.



CONCLUSIONS AND FUTURE DIRECTIONS

- **Conclusions:**
  - We were able to purify HL673 mAb using protein A affinity chromatography
  - The purified antibody was active and could bind to turtle serum proteins
  - The biotinylated HL673 was also active and could bind to turtle serum proteins
  - We could detect B cells in the spleens of adult and hatchling turtles using this reagent.
- **Future Directions:**
  - Our first experiments indicate that hatchling turtles may have a higher percentage of B cells in their spleen than adults. This needs to be repeated.
  - Using this HL673-biotin, we can label and purify B cells from blood or other tissue samples
  - We will also investigate alternative methods of B cell detection, such using the protein PAX5.

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