Curriculum Map 2—Alignment of Biology program learning outcome with USF institutional learning outcomes.

	PLO 1— Demonstrate both in-depth and broad knowledge of the concepts that comprise the biological sciences.	PLO 2—Apply the scientific process, including designing and conducting experiments and testing hypotheses.	PLO 3— Demonstrate proficiency in laboratory techniques.	PLO 4—Demonstrate the ability to read, understand, and critically review scientific papers and prepare oral and written reports in a standard scientific format.	PLO 5—Demonstrate an awareness of the significance ethics plays in the biological sciences.
ILO 1—Students reflect on and analyze their attitudes, beliefs, values, and assumptions about diverse communities and cultures and contribute to the common good.					
ILO 2—Students explain and apply disciplinary concepts, practices, and ethics of their chosen academic discipline in diverse communities.	X	X		X	X
ILO 3—Students construct, interpret, analyze, and evaluate information and ideas derived from a multitude of sources.	X	X		X	X
ILO 4— Students communicate effectively in written and oral forms to interact within their personal and professional communities.				X	X
ILO 5— Students use technology to access and communicate information in their personal and professional lives.		X	X	X	
ILO 6— Students use multiple methods of inquiry and research processes to answer questions and solve problems.		X	X	X	X
ILO 7— Students describe, analyze, and evaluate global interconnectedness in social, economic, environmental and political systems that shape diverse groups within the San Francisco Bay Area and the world.					

Appendix A: Human Anatomy Lab—Assessment of Dissection (Fall 2015)

What was done?

We evaluated dissection skills in BIOL 366-Human Anatomy Lab. Students worked in groups of three to produce a video demonstrating dissection of 3-4 muscles in a preserved cat and showing 1) knowledge of safety, 2) proper use of dissection tools, and 3) techniques for dissecting muscles. Videos were evaluated by a panel of three faculty members in the Biology Department with experience in teaching anatomy and physiology. Student groups were rated as "skilled" if they demonstrated a thorough and detailed knowledge of safety and tool use and cleanly isolated muscles from origin to insertion. Groups were rated as "proficient" if they demonstrated a complete but not detailed knowledge of safety or did not fully demonstrate clean and precise use of tools or did not fully isolate muscles from origin to insertion. Groups were rated as "not proficient" if they did not completely describe safety considerations or tool use or did not completely or cleanly isolate all of the muscles they dissected. The rubric for evaluating videos is included below.

What was learned?

All seven student groups were able to dissect they list of muscles they were given. Four of the seven groups were rated as "skilled," two were rated as "proficient," and one was rated as "not proficient," primarily for lack of thoroughness and completeness. The ratings were unanimous among the reviewers.

What changes will be made?

Production of the videos provided a valuable learning opportunity for students. Safety knowledge and dissection skill improved over the course of making the videos. Assignments involving production of dissection videos will be incorporated into the class to increase hands-on learning. Evaluation of video assignments will also provide a means for identifying deficiencies in students' dissection skill and knowledge of safety, and for providing feedback to students.

Rubric for Evaluating Dissection Videos

Skilled	The objectives of muscle dissection are clearly articulated. Safety considerations are thoroughly discussed. Use of tools is discussed, and the correct tools are used for each element of the dissection. Muscles are correctly identified and cleanly and completely separated from near the origin to near the insertion. Muscles that need to be reflected are carefully cut.
Proficient	The objectives of muscle dissection and safety considerations are mentioned. Dissection tools are discussed, and the correct tools are generally used in each element of the dissection. Muscles are correctly identified. Muscles are generally isolated from near origin to near insertion when possible, and reflected when they should be.
Not Proficient	The objectives of muscle dissection, safety considerations, or use of tools is not fully discussed. Incorrect tools are used in specific elements of the dissection. Muscles are incorrectly identified, incompletely isolated, cut into separate pieces, or not reflected when they should be.

Assessment of Student Work

Lab Notebooks from BIOL 346/347 General Microbiology Fall 2015

Faculty Member Evaluating Work:

Student Name	Assessment	Comments
Patrick Boada	Excellent	Very professional-looking.
Kris Bolfango	Excellent	Well presented with clear descriptions.
Kandace Booth	Good	Clear descriptions of procedures.
Xuewen Hong	Good	Early descriptions were vague, but became much more thorough later.
Drew-Joseph Noma	Good	Toward the end descriptions appeared to be mimicking procedures from a manual rather than describing what was actually done.
Niecholle Roco	Excellent	Clear, thorough descriptions.
Alec Starzinski	Excellent	Descriptions were thorough and easy to follow.

Rubric for lab notebooks

Excellent The student meets all expectations and work is of very high quality.

- The student has an entry for each item on the list (see attached template with List/Table of Contents) and each entry is dated.
- Organisms and specimen are clearly identified.
- Entries contain enough information to replicate the experiment, including type of culture media, incubation temperature and time, source of inoculum, magnification for microscope images and any stain or fixation procedure used.
- The outcome of the experiment is clearly described and the evidence for the result is given (ex: The culture was determined to contain *E. coli* because streaking on eosin methylene blue agar resulted in a metallic green growth that is characteristic of *E. coli.)*

Good The student adequately meets expectations and work is of good quality.

- The student has an entry for each item on the list, most organisms and specimen are identified.
- Entry contains adequate information to understand the experiment.
- Results are described but evidence supporting the conclusions are not explicitly stated or clearly noted.

Inadequate The student does not meet minimum expectations for a lab notebook.

- The student does not have an entry for each item. Entries are not dated.
- Entries lack enough information to replicate the experiment. Important information (incubation time, temperature, type of medium, type of inoculum) is missing.
- Results are not clearly described, supporting evidence is not noted.
- Organisms or specimen are not clearly identified.

Outline Provided to Students:

Use the above for your cover sheet, then add your content (please include a table of contents with page numbers, and then on a new page insert a picture of each of the following; be sure to include text with date and description of image, including magnification for microscope images and incubation conditions, such as temp, time for culture plates):

1. Threads showing depth of field
2. Image of wet mount (your choice – be sure to describe specimen, magnification, etc).
3. Image of prepared slide (your choice – be sure to describe specimen, magnification, etc).
4. Simple stain of bacilli
5. Image of culture plate from Ubiquity Exercise
6. SEM or confocal image of specimen of your choice (use internet and cite source, describe specimen and method)
7. Gram stain containing both gram positive cocci and gram negative rods in one field
8. Gram stain of your unknown from lab 4 – give number and identity
9. Image of your bacteriophage plaque assay (hint: line up plates and take one picture).
10. Table of results from bacteriophage plaque assay with calculated PFU/ml
11. Image of culture plate showing isolation streak
12. Image of MSA plate
13. Image of EMB plate
14. Image of Blood agar plate
15. Gram Stain from Lab 5 Mixed culture
16. Gram Stain from Lab 5 Unknown
17. Image of TSI tubes
18. Image of SIM tubes (with indole test)
19. Image of Citrate Slants
20. Image of Urease tubes
21. Image of Kirby-Bauer Test for *E. coli*
22. Image of Kirby-Bauer Test for *S. aureus*
23. Images of unknown organisms test results (clinical lab identification)
24. Image of beer brewing, apparatus
25. Image of raw chicken plates
26. Image of raw hamburger plates

27. Image of bacterial count plates, including table or results including CFU/ml (similar to bacteriophage lab)
28. Image of restriction digest gel – labeled
29. Image of PCR gel – labeled
30. Image of transformation results

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Hemopoietic tissue volume is a heritable trait in the schistosome-transmitting snail *Biomphalaria glabrata*

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Abstract. The anterior pericardial wall or amebocyte-producing organ (APO) is a site of hemocyte formation in the schistosome-transmitting snail *Biomphalaria glabrata*. Histological sections of the APOs of adult schistosome-resistant Salvador strain snails, and two schistosome-susceptible M-line strains, BRI-M and USF-M, showed qualitatively differing amounts of hemopoietic tissue (HT), with Salvador>BRI-M>USF-M. Adult Salvador snails also had a significantly higher concentration of hemocytes in the hemolymph than the two M-Line strains. In juvenile snails of the same three strains, measurements of total APO HT volume confirmed the qualitative differences seen in adults, and differences between the three strains were statistically significant. Crosses between Salvador (large HT volume) and USF-M (small HT volume) show that a large HT volume is dominant in juvenile F_1s. Analysis of the distribution of HT volume among F_2s shows it to be a quantitative trait. Although USF-M juvenile F_0s had a significantly lower APO HT volume than that of BRI-M F_0s, they had a significantly higher mitotic index, possibly as a compensatory mechanism. Salvador APO allografts maintained HT volume and mitotic activity equally well in USF-M and Salvador recipients after 2 weeks, suggesting that the low HT volume in USF-M snails may result from a developmental defect rather than a lack of HT-supportive plasma factors.

Additional key words: amebocyte-producing organ, hemopoieis, hemocyte, mollusk

Schistosoma mansoni Sambon 1907, an etiologic agent of the disease schistosomiasis, utilizes *Biomphalaria glabrata* Say 1818 as an obligate intermediate host, in which it undergoes asexual reproduction and development to the human-infective cercaria stage. Upon invasion of the snail by the miracidium, successful infection is dependent on compatibility between parasite and host genotypes, and a key determinant of this compatibility is the outcome of the interaction between the larval schistosome sporocyst and the humoral and cellular components of the host internal defense system (IDS), which is capable of killing incompatible parasites (Yoshino & Coustau 2011). The effector cell in IDS-mediated killing is the hemocyte or amebocyte, which rapidly encapsulates, disrupts, and phagocytoses incompatible sporocysts (Loker et al. 1982).

As reviewed by Pila et al. (2015), *B. glabrata* hemocytes are centrally produced in hemopoietic tissue (HT) within the anterior wall of the pericardial sac, a structure designated as the amebocyte-producing organ or APO (Lie et al. 1975). Several studies have described increased cell proliferation in this HT following challenge with larval trematodes or with several types of non-self substances, as well as adoptive transfer of resistance by APO allografts (Pila et al. 2015). Injection of pathogen-associated molecules elicits increased expression of genes in the APO involved in cell proliferation, immune responses, and detoxification (Zhang et al. 2016). Thus, the APO, although not directly involved in parasite killing, is an integral component of the snail IDS.

The impetus for this study was the observation during prior work that the APO of adults of the schistosome-susceptible M-line strain of *B. glabrata* (Newton 1955) maintained at the University of San Francisco (USF-M) responds weakly or not at all to stimuli that are mitogenic in other snail strains. Moreover, histological sections of the pericardial sac of USF-M snails have usually shown only rudimentary or in some specimens no HT in the APO, whereas other laboratory strains of *B. glabrata* possess abundant HT. In the present work we explored

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a possible genetic basis for this deficiency, and its relationship with HT mitotic activity and circulating hemocyte concentrations. In addition, we used APO allografts to test whether the atrophic APO in USF-M snails resulted from a lack of HT growth or maintenance factors in the plasma.

Methods

Snails

Three inbred strains of *Biomphalaria glabrata* were used: Salvador, USF-M, and a second M-line strain obtained from the Biomedical Research Institute (Rockville, MD, USA), here designated BRI-M. Salvador snails are pigmented and are refractory to infection (Paraense & Correa 1963), whereas M-line snails are albinos (hence appearing red due to hemoglobin) and are generally susceptible to infection with *Schistosoma mansoni* (Newton 1955), except for the NIH-Sm-PR2 strain (Sullivan & Richards 1981). However, it is now known that M-line snails show significant genetic diversity within and among populations from different laboratories, possibly resulting from genetic drift, mutations, and contamination with other snail strains (Mulvey & Bandoni 1994). Consequently, the precise relationship of USF-M and BRI-M to each other and to the original M-line snail developed by Newton (1955) is unknown. During this study, snail strains were maintained at room temperature (23–25°C) in separate aerated, 10-gallon aquaria containing limestone gravel and artificial pond water (Malek & Cheng 1974), and were fed Romaine lettuce *ad libitum*.

APO size in adult Salvador, USF-M, and BRI-M

To confirm prior subjective observations of differences in the amount of HT in the three snail strains, the pericardial sac was dissected from 10 each Salvador, USF-M, and BRI-M adult snails (11–12 mm, shell diameter) reared in aquaria (see Zhang et al. 2016 for an example of this dissection). Pericardial sacs were fixed in 1/3 Bouin's fluid at 50°C, dehydrated in an isopropanol-xylene series, embedded in paraffin, and serially sectioned at 7 μm. Sections were stained with Delafield's hematoxylin and eosin. The relative amount of HT in the APO of each strain was then assessed qualitatively.

Hemocyte concentrations in adult Salvador, USF-M, and BRI-M

To ascertain whether differences in amounts of HT in adult APOs were associated with different concentrations of circulating hemocytes, hemolymph was obtained from 10.5 to 13 mm aquarium-reared snails by puncturing the body wall on the left side through a hole in the shell, causing hemolymph to pool in the shell depression (Jeong et al. 1980). A 2-μL volume was transferred from the shell depression to a poly-l-lysine coated microscope slide, which was placed in a humidity chamber at room temperature for 5 min to allow the cells to settle and attach. An 18-mm circular cover slip was then lowered onto the hemolymph, and all adherent cells in the sample were counted with the use of a phase contrast microscope equipped with an eyepiece counting grid and a 20x objective. To facilitate locating hemocytes, the hemolymph was placed in the center of an approximately 7-mm diameter ring made on the underside of the slide by wetting the open end of a 200-μL micropipette tip with ink from a marking pen and pressing it against the slide.

Isolation of F_0 juvenile snails of the three strains

We studied the inheritance of HT volume and mitotic activity in juvenile snails rather than in adults because large numbers of juveniles can be raised quickly in small containers. The overall scheme for this portion of the study is shown in Fig. 1. To obtain F_0 juveniles from each of the strains, ~25-cm^2 pieces of Styrofoam were placed in the aquaria containing adults. Once egg clutches had been deposited on the Styrofoam, it was transferred to 500-mL jars containing artificial pond water. Snails that hatched were raised at room temperature on a diet of boiled lettuce until they attained a size of 5.5–6 mm, whereupon pericardial sacs from 30 specimens of each strain were fixed and serially sectioned at 10 μm.

Isolation of F_1 juvenile snails from USF-M×Salvador crosses

For genetic crosses, 10 each adult (12–13 mm) Salvador and USF-M snails from aquaria were paired in 500-mL jars for 3 weeks. Subsequently, the Salvador parent was discarded, eggs on the surface of the jars or USF-M shells were removed, and the USF-M parent was allowed to lay egg clutches on pieces of Styrofoam placed in the jar. These eggs were transferred to new jars to produce F_1 hybrids, identifiable by their black eye pigmentation, a monogenic dominant trait (Richards 1970), as well as albino progeny that presumably resulted from self-fertilization with autologous sperm. Among the 10 crosses, only eight isolated USF-M parents laid

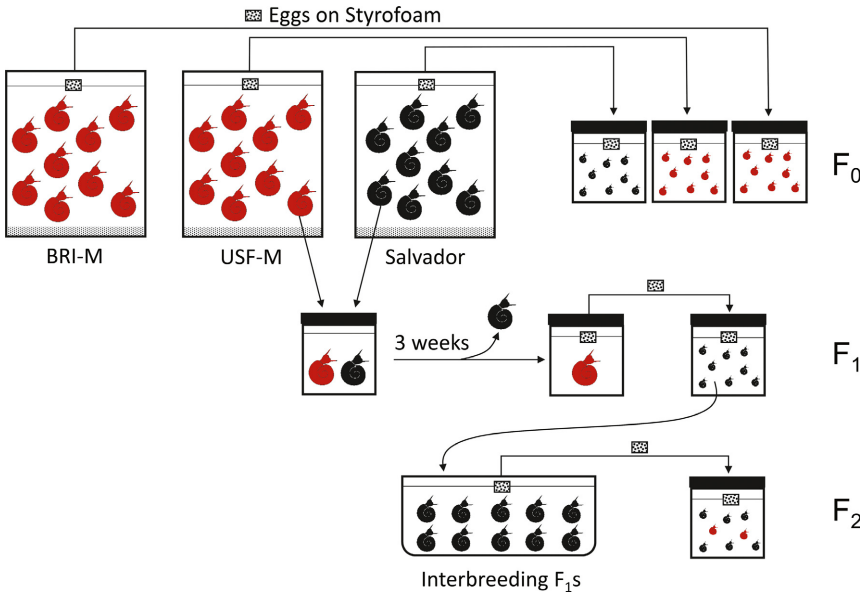

Fig. 1. Protocol for obtaining F_0, F_1, and F_2 *Biomphalaria glabrata*. F_0 snails of each strain were raised in 500-mL jars from eggs deposited by an aquarium population. F_1 snails were obtained from individual USF-M snails that had been mated with a Salvador snail for 3 weeks. F_2 snails were obtained from interbreeding F_1 hybrids in 2-L containers.

eggs, and of these, two produced no hybrid (pigmented) offspring, evidently having failed to mate successfully with the Salvador partner. From each of the six successful crosses, pericardial sacs from 10 juvenile F_1 hybrid offspring were fixed and serially sectioned at 10 μm (total=60). In addition, 50 pericardial sacs were fixed and sectioned from albino (non-hybrid) F_1 offspring of the seven USF-M parents that produced such progeny (4–10 pericardial sacs from each parental line), in order to compare them with the USF-M F_0 snails.

Isolation of F_2 juvenile snails

To produce F_2s, 10 F_1 hybrids from five of the six successful crosses were pooled in separate plastic rectangular covered containers containing approximately 2 L of water. In the case of the sixth cross, only four F_1 hybrids were available, but these produced abundant egg clutches. Egg clutches deposited by these pooled F_1s on Styrofoam or scraped from the sides of the container were transferred to 500-mL jars to produce F_2 progeny. Jars were examined every 2–3 d, and pericardial sacs were fixed from the first snails in each jar to attain 5.5–6 mm (34–38 F_2s from each of the six parental lines, 217 total). Of these, 10 pericardial sacs from each of the six lines were used for volume measurements, the rest serving as replacements for damaged histological specimens and for calculation of pigmented:albino phenotypic ratios.

Measurement of HT volume and mitotic activity in F_0, F_1, and F_2 juvenile snails

Accurately measuring the amount of HT in the APOs of different snails by simple visual inspection of histological sections, as performed with adult snails above, can be problematic because of the uneven distribution of HT tissue within the APO, tissue distortion resulting from dissection and fixation, and the variable orientation of the APO with respect to the plane of section. Consequently, sectioned HT can appear either patchy or continuous, and in profiles ranging from narrow (sagittal section) to wide (tangential section). Therefore, we devised a standard method for quantifying total HT volume in individual APOs. Serial 10-μm-thick sections of pericardial sacs from juvenile snails were first scanned at low magnification in order to identify the sequence containing HT, identifiable by its location and basophilic staining. All sections of the APO containing HT were then individually photographed as JPEG files with an Insight digital camera (Diagnostic Instruments, Sterling Heights, MI) mounted on a Nikon E600 microscope. For each APO, magnification was adjusted with the 10x or 20x objective and a microscope magnification changer (1x, 1.25x, 1.5x, and 2x settings) so that the largest section of HT in the specimen could be included in a single rectangular frame, after rotating the camera as necessary; that magnification was then used for all other sections of the APO. Using

ImageJ image processing software (www.imagej.nih.gov), the perimeter of HT in each photograph was manually traced on a computer monitor with a stylus and an Intuos PTH-850 pen tablet (Wacom, Vancouver, WA, USA). Perimeter tracings were converted into surface area (μm^2) by ImageJ, following scale calibration with an image of a stage micrometer captured at the same magnification. The sum of the surface area measurements for all sections of a specimen multiplied by the section thickness (10 μm) gave the HT volume of the APO in μm^3.

For comparing mitotic activity, the number of histological sections of HT in F_0, F_1, and F_2 snails (7407 HT sections from 260 APOs) made it impractical to count all mitotic figures, particularly in 10-μm sections having multiple cell layers. Instead, total mitotic figures were counted in the single section of each specimen having the largest measured surface area, using a 100x oil immersion objective. These counts were then normalized for tissue volume of the section to give a volumetric mitotic index, expressed as mitotic figures mm^{-3}. In instances where no mitotic figures were observed in the largest section, additional adjacent sections were examined until one or more figures occurred, and mitotic figures mm^{-3} were then calculated based on the sum of all the examined HT section volumes. Snails that lacked HT were assigned a mitotic index of 0.

Transplantation of the APO

In order to explore possible differences in HT growth or maintenance factors between Salvador and USF-M plasma, APO allografts were transplanted heterotopically from Salvador donors (10–10.5 mm shell diameter) into either 10 Salvador or 10 USF-M recipients (12–13 mm, shell diameter). All of these snails had been raised in aquaria. The donor APO was dissected from the pericardial sac in 1/3 phosphate-buffered saline (PBS), rinsed for ~1 min in fresh PBS, and implanted into a hemolymph sinus anterior to the digestive gland on the left side, as described previously (Sullivan 1990). A piece of stretched polyethylene bubble wrap and a coating of rubber cement were used to seal the implant wound. Recipients were maintained in individual jars at 27°C for 1–2 weeks, after which they were dissected and the implant was fixed and serially sectioned at 7 μm. Controls consisted of 10 unimplanted APOs from aquarium-reared Salvador snails. HT volume of each implanted and control APO was measured in histological sections, as described above. In addition, the total number of mitotic figures in all sections of HT in each implant was counted.

Statistical analysis

Post-hoc comparisons of HT volumes and mitotic counts in juvenile snails were performed by multiple pairwise comparisons with the two-tailed Student's *t*-test. Type-I statistical error was controlled by adjusting significance thresholds for the number of comparisons made, with the use of the sequential Bonferroni method (Rice 1989). HT volumes and mitotic counts of implanted Salvador APOs in USF-M or Salvador recipients were compared with those of unimplanted Salvador APOs with the two-tailed Student's *t*-test. Differences were considered statistically significant at $p < 0.05$.

Results

Comparison of APOs in adult snails

Sagittal sections of the APO of adult Salvador snails showed a confluent mass of HT of varying width (Fig. 2A). In the case of USF-M snails, at most only a single small nodule was found (Fig. 2B), and three of the 10 snails examined possessed no HT in the APO. Much of the interior of the APO in adult USF-M snails contained numerous concretions in the region where hemopoietic cells would normally be found (Fig. 2C). These concretions appeared as glistening spherical bodies upon dissection of the pericardial sac but were reduced to irregular membranous structures after fixation in Bouin's fluid, hence were probably composed of $CaCO_3$. Finally, the APO of BRI-M snails seemed to possess a moderate amount of HT in a confluent mass, somewhat less than in Salvador snails.

Comparison of hemocyte concentrations in adult snails

In addition to a larger amount of HT, adult Salvador snails had significantly higher numbers of hemocytes in the hemolymph than did USF-M or BRI-M snails (Fig. 3). However, whereas adult BRI-M adults appeared to possess much more HT than USF-M snails, hemocyte counts in the latter were slightly higher.

HT volumes in juvenile F_0 snails

As expected on the basis of the above histological observations in adults, juvenile F_0 Salvador snails

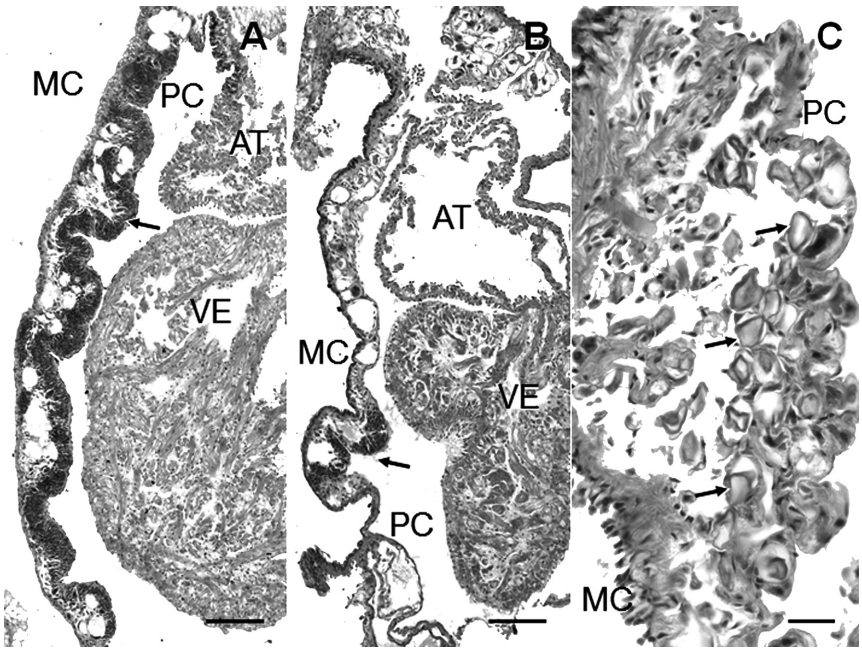

Fig. 2. Representative sagittal histological sections of the amebocyte-producing organ (APO) of adult *Biomphalaria glabrata*. **A.** Salvador APO, containing a confluent mass of basophilic hemopoietic tissue (arrow); **B.** USF-M APO with a small nodule of hemopoietic tissue at arrow; **C.** Higher magnification of a region of USF-M APO, showing large concretions (arrows) filling the interior. AT, atrium; MC, mantle cavity; PC, pericardial cavity; VE, ventricle. Scale bars: A, B=100 μm; C=20 μm.

possessed the highest HT volume in the APO among the three strains (Fig. 4). USF-M F_0 snails had the smallest volume, with two of 30 snails having none. There was no overlap between the range of values in Salvador (1.42×10^6–4.63×10^6 μm^3) and USF-M (0–1.39×10^6 μm^3) snails. BRI-M F_0 snails had an intermediate HT volume. Differences between the mean values in the three strains were statistically significant.

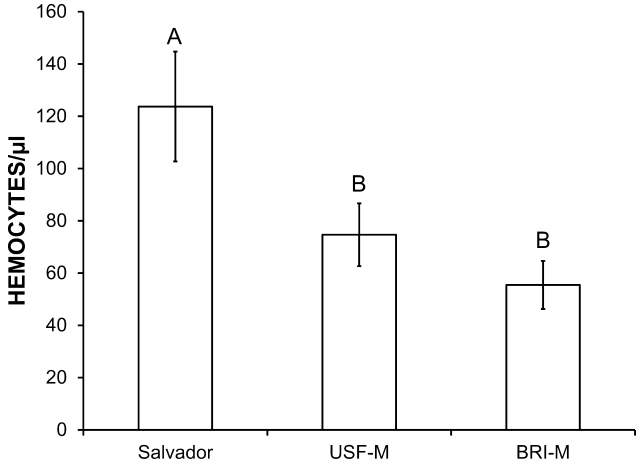

Fig. 3. Concentrations of hemocytes in the hemolymph of adult Salvador, USF-M, and BRI-M *Biomphalaria glabrata*. Differences between bars marked with different letters are statistically significant ($p < 0.05$). Error bars indicate standard error; n=30.

Genetic crosses

Hybrid (pigmented) F_1s produced by USF-M parents inseminated by Salvador snails had an HT volume that was nearly identical to that of Salvador F_0 snails (Fig. 4). Conversely, non-hybrid (albino) F_1s had a small HT volume, similar to that of USF-M F_0 snails, indicating that the rearing conditions of the parent (isolation in jar vs. aquarium) and the mechanism of fertilization (self vs. cross) did not significantly affect the amount of HT in the progeny. Among 50 non-hybrid F_1s measured, two possessed no HT in the APO.

Among the 217 juvenile F_2s that were scored for pigmentation, 170 pigmented and 47 albinos occurred, giving a ratio of 3.62:1. Average HT volume in 60 of these F_2s was slightly less than one half of Salvador F_0 snails, but was still significantly higher than in USF-M F_0 and albino F_1 snails (Fig. 4). When the total range of F_2 volume measurements (0.129×10^6–2.83×10^6 μm^3) was divided into quintiles and plotted in a histogram, values showed a continuous distribution (Fig. 5).

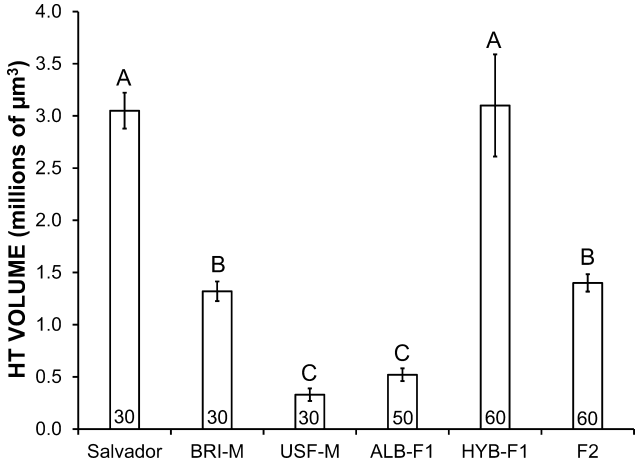

Fig. 4. APO hemopoietic tissue volumes in juvenile Salvador, BRI-M, USF-M, non-hybrid albino (ALB) F_1, hybrid (HYB) F_1, and F_2 *Biomphalaria glabrata*. Differences between bars marked with different letters are statistically significant ($p < 0.05$). Numbers of APOs measured are shown at the base of each bar. Error bars indicate standard error.

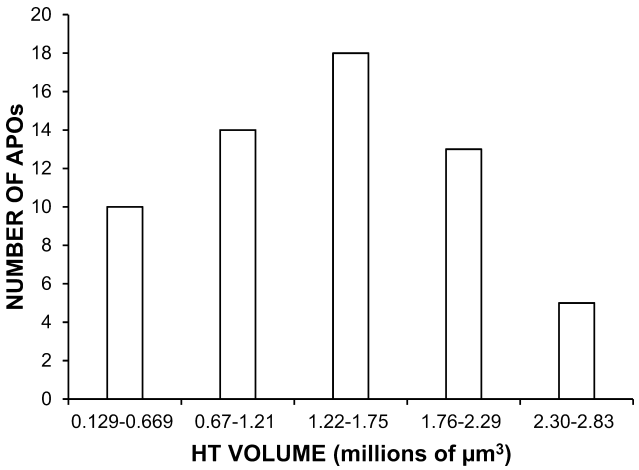

Fig. 5. Histogram showing the continuous distribution of APO hemopoietic tissue volumes in 60 juvenile F_2 *Biomphalaria glabrata*.

Mitotic activity in USF-M, BRI-M, Salvador, F_0, F_1, and F_2 snails

Although HT volume in USF-M F_0 and non-hybrid F_1 snails was significantly lower than that in all other groups (Fig. 4), paradoxically the APO mitotic index was higher, and this difference was statistically significant with respect to BRI-M and hybrid F_1s (Fig. 6).

APO implants

At both 1 and 2 weeks post implantation, HT volume and mitotic activity of Salvador APO implants in USF-M recipients were unchanged compared to pre-implant controls (Figs. 7, 8). By comparison, in homologous Salvador recipients, while implant HT volume and mitotic activity increased slightly at 1 week post implantation, both parameters were lower at 2 weeks, and the decrease in HT volume was statistically significant.

Discussion

Previous descriptions of the amount of HT in the APO have relied on measurement of maximum width in sagittal sections (Noda 1992), or morphometric measurements of randomly selected sections (Souza & Andrade 2006). By comparison, the method used in this study captured the entire volume of HT in the APO of individual snails. This technique has the further advantage of requiring relatively simple equipment (a microscope with a digital camera, computer, and pen tablet) and publically available image analysis software (ImageJ).

The inter-strain differences in the amount of HT and hemocyte concentrations in adults and in HT volumes among juvenile F_0 snails indicate a genetic basis for these results. Indeed, the crosses of Salvador and USF-M parents demonstrate that large HT volume is inherited as a dominant trait in hybrid F_1s (Fig. 4).

The continuous distribution of HT volumes in F_2s is characteristic of a quantitative trait, in other words, one controlled by multiple genes (Fig. 5).

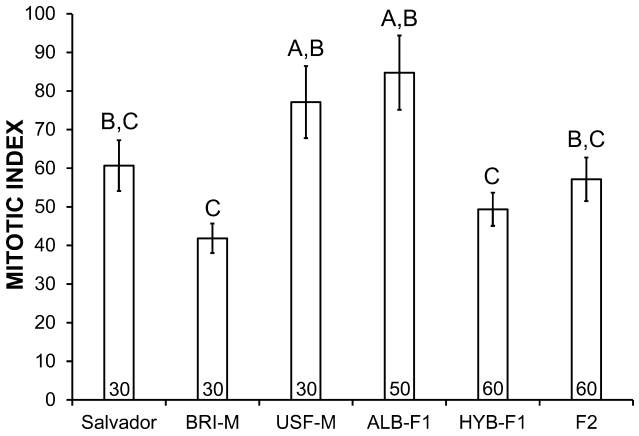

Fig. 6. Volumetric mitotic index (mitotic figures mm^{-3}) of Salvador, BRI-M, USF-M, non-hybrid albino (ALB) F_1, hybrid (HYB) F_1, and F_2 *Biomphalaria glabrata*. Total mitotic figures in the largest 10-µm section(s) were counted and normalized for tissue volume. Differences between bars marked with different letters are statistically significant ($p < 0.05$). Numbers of APOs measured are shown at the base of each bar. Error bars indicate standard error.

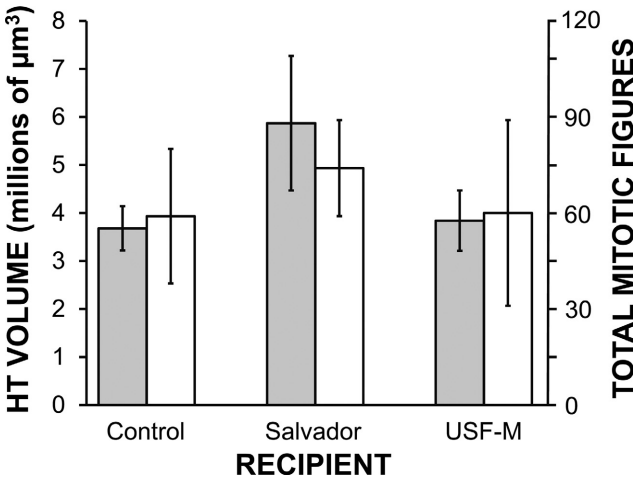

Fig. 7. Hemopoietic tissue (HT) volumes (shaded bars) and numbers of mitotic figures (unshaded bars) in Salvador APO allografts at 1 week post implantation into Salvador or USF-M *Biomphalaria glabrata* recipients. Controls show values in unimplanted APOs. Error bars indicate standard error; n=10.

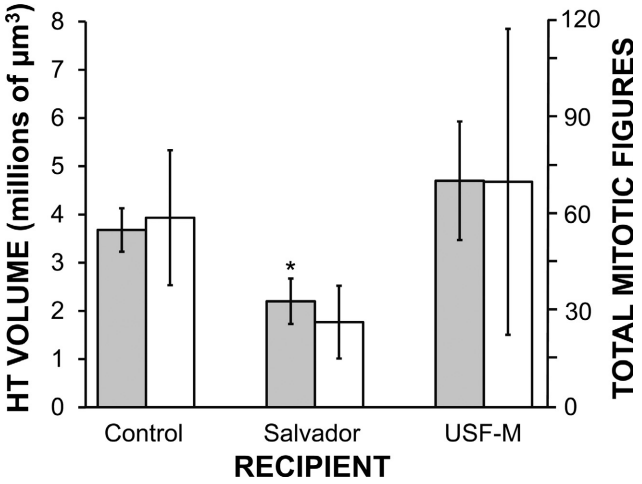

Fig. 8. Hemopoietic tissue (HT) volumes (shaded bars) and numbers of mitotic figures (unshaded bars) in Salvador APO allografts at 2 weeks post implantation into Salvador or USF-M *Biomphalaria glabrata* recipients. Controls show values in unimplanted APOs (same data as in Fig. 7). *Significantly lower than preimplant control ($p<0.05$). Error bars indicate standard error; n=10.

The nearly identical volumes in Salvador F_0s and hybrid F_1s suggest that Salvador F_0 snails are mostly homozygous for the dominant allele of relevant genes. The lower average value of HT volume in F_2s, which is less than 50% of that in Salvador F_0 snails, implies homozygous recessive genotypes in one or more loci (expected at a frequency of 0.25 for any one gene, assuming heterozygous F_1s) causing a reduction in the value of the trait.

USF-M F_0 and non-hybrid F_1 snails have a significantly higher mitotic index than BRI-M F_0 snails, despite a significantly lower volume of HT. This increased rate of cell division may be a compensatory mechanism to maintain homeostasis of hemocyte numbers in the hemolymph.

Our results raise the question of the origin of hemocytes in adult USF-M snails, which lack a well-developed APO and yet have hemocyte concentrations slightly higher than those in BRI-M snails, which possess much more HT both as juveniles and adults. Some of these hemocytes may have been formed during the juvenile stage, when mitotically active HT is present in USF-M snails, and then persisted into adulthood. However, since invertebrate hemocytes are believed to live for only days to weeks (Accorsi et al. 2014), this explanation is unlikely. Instead, hemopoiesis in adult USF-M snails is probably occurring in a location outside of the APO, for example, in the hemolymph, kidney, head foot, or connective tissue of the mantle, which are additional sites for hemocyte production in gastropods (Pila et al. 2015).

The spherical bodies observed in the anterior pericardial wall of adult USF-M snails, as discussed above, appear to be calcareous in composition, since they collapse following fixation in acidic Bouin's fluid. Pan (1958) refers to "crystalline concretions" as a normal component of the connective tissue of *Biomphalaria glabrata,* and Haley & Gibson (1971) describe "calcium spherules" functioning as sites of calcium storage in the connective tissues of the mantle and in the head foot of *Helisoma duryi* (Wetherby 1879). The reason for their accumulation in the anterior pericardial wall of adult USF-M snails, and whether their presence is causally related to the lack of HT is not known.

Adult Salvador snails have approximately 1.5- to 2-fold more hemocytes compared to susceptible USF-M and BRI-M snails, respectively, as well as a larger APO that presumably accounts for this difference, and whether the higher cellularity of the IDS itself contributes to schistosome resistance is unclear. On the one hand, higher numbers of hemocytes might produce a more robust encapsulation and cytotoxic response against invading sporocysts, and interestingly a higher concentration of hemocytes in schistosome-resistant versus schistosome-susceptible strains of *B. glabrata* has been reported previously (Coustau & Yoshino 1994). However, many investigators also have shown qualitative and quantitative differences in functional characteristics between hemocytes of resistant and susceptible snails that may be relevant to sporocyst recognition,

adherence, and killing. These include, but are not limited to, differences in lectin-binding specificities (Schoenberg & Cheng 1980; Martins-Souza et al. 2006), lysosomal enzyme content (Granath & Yoshino 1983), cell surface antigens (Granath & Aspevig 1993; Agner & Granath 1995), cell surface polypeptides (Coustau & Yoshino 1994), production of hydrogen peroxide following stimulation by phorbol myristate acetate (Bender et al. 2005), levels of cytosolic superoxide dismutase (Bender et al. 2007), patterns of gene expression following exposure to *Schistosoma mansoni* or its excretory-secretrory products (Lockyer et al. 2008; Zahoor et al. 2014), and glycans shared with *S. mansoni* (Yoshino et al. 2013).

A novel explanation that reconciles the roles of the above two factors in resistance to schistosome infection—hemocyte concentration and functional characteristics—has been proposed by Larson et al. (2014), who show a correlation between numbers of spread hemocytes and resistance in 52 inbred lines of the 13-16-R1 strain of *B. glabrata*. These authors hypothesize that high cell numbers are sufficient for resistance, whereas in snail lines with low cell numbers, resistance depends upon expression of immune-related genes. Thus, Salvador snails may owe their resistance in part to increased numbers of hemocytes produced by a large APO, whereas M-line snails, with lower numbers of hemocytes and a smaller APO, are susceptible due to failure to express appropriate immune-related genes.

The ratio of pigmented to albino F_2s (3.62:1) differed from the expected 3:1 Mendelian ratio. Although this discrepancy could be due to the small sample size, Vianey-Liaud et al. (1996), using a much larger number of specimens (6208, based on embryos in egg clutches), also found a 3.63:1 of black-eyed to albino offspring from heterozygous *B. glabrata*. These investigators hypothesized that the departure from the expected 3:1 ratio resulted from competition between sperm, with those carrying the dominant pigmentation allele being longer and perhaps faster swimmers.

Transplantation studies showed that implanted Salvador APOs survived and maintained HT volume and mitotic activity in USF-M recipients as well as or even better than (i.e., at 2 weeks) they did in the homologous Salvador recipient. These results, combined with the observation of a high mitotic index in juvenile USF-M snails, lead us to conclude that the relatively atrophic APO in USF-M snails probably is not due to insufficiency of plasma factors required for HT maintenance and mitotic activity. Instead, results may point to a developmental defect in either the hemocyte precursor cells that normally populate the APO or in the components of the APO stroma (e.g., fibroblasts, pore cells, muscle cells, and extracellular matrix) (Pila et al. 2015), that may provide a necessary microenvironment for HT function and that seem to be largely replaced by calcium concretions in adult USF-M snails.

Conclusions

In this study, we have demonstrated a method for measuring the size of a component of the IDS of *Biomphalaria glabrata*. Overall, our results suggest that the significantly larger volume of HT in Salvador *B. glabrata* is a heritable, dominant, and polygenic trait. The larger amount of HT and higher concentrations of hemocytes in Salvador snails may be partly responsible for their resistance to infection with *Schistosoma mansoni*. The cause of the small volume of HT in the APO of USF-M snails is unknown, but may involve a developmental defect.

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Effects of abnormal temperature and starvation on the internal defense system of the schistosome-transmitting snail *Biomphalaria glabrata*

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ABSTRACT

Climate change may affect the internal defense system (IDS) of freshwater snails, and as a result their capacity to transmit disease. We examined effects of short-term exposure to supra- and sub-optimal temperatures or starvation on 3 parameters of the IDS of the schistosome-resistant Salvador strain of *Biomphalaria glabrata* — hemocyte concentrations, cell division in the amebocyte-producing organ (APO), and resistance to infection with *Schistosoma mansoni*. Adult snails were exposed to 1 of 3 temperatures, 20 °C, 27 °C (controls), or 33 °C, for 1 or 2 weeks, with food. A fourth group was maintained at 27 °C, but without food. Compared to the controls, starved snails had significantly higher hemocyte counts at both 1 and 2 weeks, although mitotic activity in the APO was significantly lower at both time periods. Exposure to 20 °C or 33 °C for 1 or 2 weeks did not affect hemocyte numbers. However, APO mitotic activity in snails exposed to 20 °C was significantly higher at both 1 and 2 weeks, whereas mitotic activity in snails exposed to 33 °C was significantly lower at 1 week but normal at 2 weeks. None of the treatments altered the resistance phenotype of Salvador snails. In a follow-up experiment, exposure to 33 °C for 4–5 h, a treatment previously reported to both induce expression of heat shock proteins (Hsps) and abrogate resistance to infection, caused immediate upregulation of Hsp 70 and Hsp 90 expression, but did not alter resistance, and Hsp expression levels returned to baseline after 2 weeks at 33 °C. Results of this study indicate that abnormal environmental conditions can have both stimulatory and inhibitory effects on the IDS in adult *B. glabrata*, and that some degree of acclimation to abnormal temperatures may occur.

1. Introduction

Changes in global climate may affect the biology of disease-transmitting snails (Mas-Coma et al., 2009), presumably through effects on water temperature, water chemistry, food availability, and aquatic community structure. Several studies have attempted to predict the impact of climate change on schistosomiasis transmission (reviewed by McCreesh and Booth, 2013), and using an agent-based model incorporating effects of increasing temperature on both the snail host and larval parasite, McCreesh et al. (2015) have hypothesized that an increase in prevalence and intensity of schistosomiasis may occur in some parts of eastern Africa over the next several decades.

In *Biomphalaria glabrata*, an intermediate host of *Schistosoma mansoni* in the New World, a potential effect of climate change that could impact disease transmission is alteration of the immune or internal defense system (IDS), which largely determines the outcome of invasion by a larval trematode. The IDS of *B. glabrata* consists of both phagocytic hemocytes and plasma immune factors, and in incompatible *B. glabrata–S. mansoni* relationships, snail hemocytes recognize, then rapidly encapsulate and kill schistosome sporocysts (reviewed by Coustau et al., 2015). Hemocyte production occurs in a region of the anterior pericardial wall called the amebocyte-producing organ (APO) (Lie et al., 1975; Pila et al., 2016b), and is stimulated by an endogenous growth factor, *B. glabrata* granulin (Pila et al., 2016a). Hypothetically, environmentally-induced physiological stress could affect hemocytes, plasma factors, or hemopoiesis in the APO, and as a result host vector competence, making *B. glabrata* either less or more susceptible to infection with *S. mansoni* as well as microbial snail pathogens. For example, Ittiprasert and Knight (2012) have reported that exposure of 4–6 mm juvenile BS-90 (schistosome-resistant) *B. glabrata* to high temperature (32 °C) for 4 or more h causes upregulated expression of heat shock proteins (Hsps) 70 and 90. Moreover, this heat treatment abrogates resistance to infection with *S. mansoni*, apparently due to increased expression of Hsp

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90, as shown by reversal of this effect by geldanamycin, an inhibitor of Hsp 90.

The concentration of circulating phagocytic leukocytes is a commonly measured indicator of immune status in animals. For example, in humans prolonged and very low blood concentrations of neutrophils (chronic severe neutropenia) is associated with increased risk of microbial infection, and among several extrinsic causes of this condition are certain types of nutritional deficiencies that impair hemopoiesis (Newburger and Dale, 2013). In marine bivalves, a large number of studies have investigated effects of abnormal environmental conditions, e.g., toxicants, temperature, salinity, and starvation, on hemocyte concentrations, as well as on other parameters of the IDS, including susceptibility to infection with pathogens (partially reviewed by Raftos et al., 2014). Among gastropods, Al-Rawadeh (2010) reported a significant decrease in the number of hemocytes in *Helix aspersa* after 3 weeks of starvation. Stumpf and Gilbertson (1978) exposed *B. glabrata* to a range of temperatures, from 12 °C to 36 °C, for 3 days and observed a bell-shaped effect on hemocyte concentrations, with hemocyte numbers increasing from 12 °C to 27 °C and then decreasing at higher temperatures. Suresh et al. (1994) exposed 30 °C-acclimated *Lymnaea acuminata* and *Indoplanorbis exustus* to 20 °C, 25 °C, 35 °C, and 40 °C for up to 24 h, and found elevated cell counts at 20 °C and 25 °C after 2 and 12 h in *I. exustus* and at all time periods in *L. acuminata*. The highest temperature did not affect hemocyte numbers in *I. exustus* but in *L. acuminata* caused an increase after 2 h of exposure and a sharp decrease at 24 h. Hypothetically, the observed changes in hemocyte numbers could result from direct effects on hemocyte survival and/or distribution between hemolymph and tissues, as well as effects on hemocyte production.

The hypothesis for this study is that short-term abnormal environmental conditions will cause physiological stress, broadly defined as a condition that challenges homeostasis (Kagias et al., 2012), and that such stress may affect the IDS of *B. glabrata*. Relative to "normal" and "abnormal" environmental conditions, *B. glabrata*, which is endemic to South America, has been raised in the laboratory at temperatures ranging from 20 to 29 °C (Bruce et al., 1971), with an optimal temperature in the range of 24–28 °C (Eveland and Haseeb, 2011). Survival is limited to several days at 7 °C and only 4 h at 42 °C (Maldonado, 1967). Freshwater pulmonate snails normally feed on algae (Palmieri et al., 1978) and decaying plant matter (Rollinson, 2011), but in the laboratory *B. glabrata* grows and reproduces well on a diet of Romaine lettuce leaves. Young adult snails (10.1–13 mm, shell diameter) are able to survive without food for at least 22 days, although their O_2 consumption declines significantly (van Aardt et al., 2003).

In this study, we examined effects of 1- and 2-week exposures to supra- and sub-optimal temperatures or starvation on 3 parameters of the IDS of the schistosome-resistant Salvador (BS-90) strain of *B. glabrata*, i.e., hemocyte concentrations, mitotic activity in the APO as a measure of hemocyte production, and resistance to infection with *S. mansoni.*

2. Materials and methods

2.1. Snails

Schistosome-resistant Salvador (Paraense and Correa, 1963) and schistosome-susceptible M-line (Newton, 1955) *B. glabrata* were reared in aerated aquaria at room temperature, which varied from 22 to 25 °C during this study, and were fed a diet of Romaine lettuce. Because acquiring sufficient hemolymph from small snails may require pooling samples (Jeong et al., 1980), adult snails, measuring 10.5–13 mm, were used for all experiments in order to obtain individual hemocyte counts.

2.2. Miracidia

Livers were removed from mice experimentally infected with the NMRI strain of *S. mansoni* at the Biomedical Research Institute (Rockville, MD), chilled with refrigerant gel cold packs, and shipped overnight to the University of San Francisco (USF). Upon receipt of the livers, miracidia were harvested by a previously described method (Sullivan and Richards, 1981).

2.3. Exposure to different environmental conditions

Salvador snails were individually exposed in 500-ml jars in incubators maintained at 20 °C, 27 °C, or 33 °C for 1 or 2 weeks, with food. Incubators were not equipped with illumination, and consequently snails were exposed in the dark. Water was brought to the appropriate temperature before snails were added. A fourth group was maintained at 27 °C, but without food. A total of 30–31 snails were used for each treatment. For this study, 27 °C with food was considered the normal or control environmental condition.

2.4. Hemocyte and mitotic figure counts

After 1 or 2 weeks of exposure to each treatment, a total hemocyte count was obtained for each snail by counting all adherent cells in a 2-μl sample of hemolymph, as described previously (Sullivan et al., 2016). The bleeding technique involved puncturing the body wall through a hole made in the shell overlying the ventral side of the digestive gland and collecting hemolymph that welled up in the shell depression (Jeong et al., 1980). After snails were bled for hemocyte counts, the pericardial sac was dissected, fixed in 1/3-strength Bouin's fluid for at least 24 h, before being dehydrated in an isopropanol-xylene series and embedded in paraffin. Tissues were then serially sectioned at 7 μm. Sections were mounted on microscope slides, and stained with Delafield's hematoxylin and eosin. Rows of stained sections were scanned at 100× to locate the section that appeared to possess the most hemopoietic tissue (identifiable by its location and staining), and then mitotic activity was estimated by counting total numbers of mitotic figures in that section and the 2 sections on either side at 1000× (5 sections/APO). Counts were obtained for 25–30 APOs from each treatment.

2.5. Exposure to miracidia

Groups of 11–15 adult *B. glabrata,* pooled in 2 L of water in covered plastic rectangular containers, were subjected to the above 4 treatments for 1 or 2 weeks, and were then individually challenged with 25 miracidia of *S. mansoni.* Incubations were set up such that 1-and 2-week treatment groups were exposed simultaneously to the same batch of miracidia. This experiment was conducted on 3 separate occasions, using 3 different batches of miracidia. Infection controls consisted of 3 groups of 15 schistosome-susceptible M-line strain adult snails that had been reared in aquaria prior to miracidial challenge. Two of the 3 control groups were exposed simultaneously with the experimental groups; the third control group was exposed to a separate batch of miracidia. Snails were exposed to miracidia overnight at room temperature in 4 ml of water in 35-mm plastic Petri dishes immediately upon removal from the treatment temperature. Unlike the procedure of Ittiprasert and Knight (2012), we did not incubate the snails overnight in distilled water containing ampicillin beforehand. Following exposure to miracidia, snails were maintained at 27 °C with food, again in the dark, until they were dissected and assessed

for the presence of daughter sporocysts. For assessing infection status, 2 methods were employed. M-line snails, and Salvador snails exposed to 27 °C, 20 °C, or starvation, were dissected from their shells, and the digestive gland was observed with a dissecting microscope for folded, cream-colored daughter sporocysts, which usually are apparent in susceptible snails by 21 days post exposure (DPE) to miracidia (Fig. 1A). In addition, all Salvador snails exposed to 33 °C, as well as M-line snails showing no external signs of infection in the digestive gland, were minced into small pieces in 0.3% NaCl, squashed between 2 microscope slides, and examined with a phase contrast microscope at 100× for the presence of motile immature daughter sporocysts (Fig. 1B) (Sullivan and Spence, 1994). A total of 32–36 surviving Salvador snails in each treatment category and 44 M-line snails were examined for infection. All but 3 M-line snails, and approximately 1/3 of Salvador snails in each treatment group were dissected beginning at 21 DPE; the rest were dissected at 28–33 DPE. We used this method because it reliably reveals prepatent infections in susceptible snails when incubated at 27 °C following miracidial exposure (Fig. 1) and avoids the repetitive testing of uninfected snails for cercarial emergence. However, retarded infections such as those described by Lewis et al. (1993), in which secondary sporocysts were first detected in the head foot at up to 10 months after exposure to miracidia, would not be detected by this method.

Once it became apparent that snails maintained at 33 °C for 1 or 2 weeks remained uninfected after challenge with miracidia (see below), we decided to employ short-term exposures to elevated temperature, similar to the method of Ittiprasert and Knight (2012). A group of 40 adult snails was incubated at 33 °C for 5–6 h, with food, and then individually exposed to 25 miracidia from a single liver. Snails were assessed for infection by removing them from their shells beginning at 30 DPE and examining the digestive gland with a dissecting microscope as described above.

2.6. Heat shock protein expression

To detect upregulation of heat shock protein expression, adult Salvador snails were incubated at 33 °C in individual 500-ml jars for 4–5 h or 2 weeks with food. After heat treatment, 3 specimens from each group, as well as 3 untreated snails removed from aquaria (controls), were dissected from their shells, snap frozen in liquid N_2, ground to a powder, and added to 1 ml TRIzol (Life Technologies) in individual microcentrifuge tubes. Total RNA was isolated according to the manufacturer's protocol and was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen). cDNA was amplified for both qualitative and quantitative RT-PCR using Hsp 90, Hsp 70, and myoglobin gene-specific primers and conditions described previously (Ittiprasert et al., 2009; Ittiprasert and Knight, 2012). Real time PCR assays of individual snails were carried out in triplicate, using Brilliant II SYBR Green QRT-PCR Master mix (Stratagene) and a CFX96 Real-Time PCR System (Bio-Rad).

2.7. Statistical analysis

Means of hemocyte concentrations and mitotic figures in snails exposed to 20 °C, 33 °C, and starvation were compared to corresponding means in snails maintained at 27 °C with food ("normal" environmental conditions) with a two-tailed Student's *t* test. Fold-differences in expression of Hsp 70 and Hsp 90, normalized for myoglobin expression, in heat shocked snails relative to controls were analyzed for statistical significance by comparing delta Ct values with the Student's *t* test (Ittiprasert and Knight, 2012). Differences at $P < 0.05$ were considered statistically significant.

3. Results

3.1. Hemocyte counts

Mean hemocyte counts in snails subjected to 20 °C, 33 °C, or starvation for 1 week were higher than those of controls, i.e., snails incubated at 27 °C with food (Fig. 2). However, this elevation was statistically significant only in starved snails. At 2 weeks, mean hemocyte counts in snails exposed to 20 °C or starvation similarly were higher than those in controls (Fig. 3). Again, a statistically significant difference was observed only in starved snails.

3.2. Mitotic activity

At both 1 and 2 weeks, mean mitotic activity in the APO of snails exposed to 20 °C was higher than in controls, and mitotic

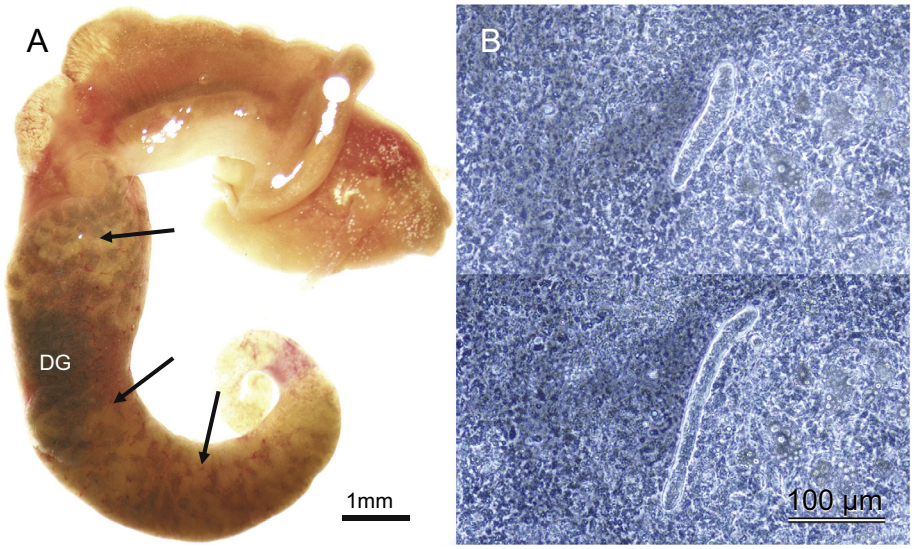

Fig. 1. Sporocysts of *Schistosoma mansoni* in *Biomphalaria glabrata*. (A) M-line snail dissected from its shell at 21 days post-exposure (DPE) to miracidia. The digestive gland (DG), which occupies most of the posterior half of the snail and is uniformly dark green in uninfected specimens, is heavily infected with cream-colored masses of secondary sporocysts (arrows). This snail also was releasing cercariae prior to dissection. (B) Phase contrast images of a motile, immature daughter sporocyst in a squash of head foot tissue from a snail at 22 DPE, showing characteristic extension and retraction of the body.

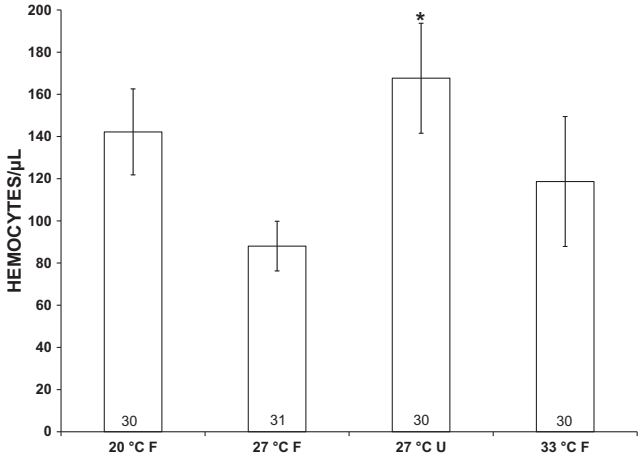

Fig. 2. Concentration of hemocytes in the hemolymph of *Biomphalaria glabrata* incubated for 1 week at 3 different temperatures and either fed (F) or unfed (U). Error bars indicate standard error. Numbers at bases of bars show sample size. *, mean is significantly different from 27 °C F treatment ($P < 0.05$).

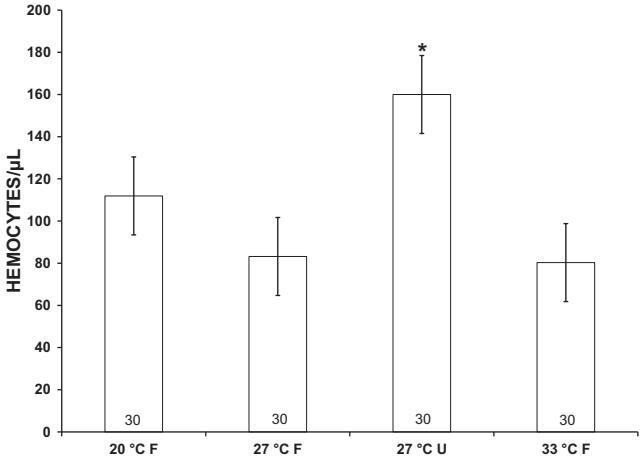

Fig. 3. Concentration of hemocytes in the hemolymph of *Biomphalaria glabrata* incubated for 2 weeks at 3 different temperatures and either fed (F) or unfed (U). Error bars indicate standard error. Numbers at bases of bars show sample size. *, mean is significantly different from 27 °C F treatment ($P < 0.05$).

activity in snails exposed to 33 °C or starvation was lower. These differences were statistically significant in snails exposed to 20 °C, 33 °C or starvation for 1 week or to 20 °C or starvation for 2 weeks (Figs. 4 and 5).

3.3. Schistosome infection

None of the experimental treatments altered prevalence of infection. Among 317 adult Salvador snails exposed for 1 or 2 weeks to 20 °C, 27 °C, 33 °C, or starvation, or exposed to 33 °C for 5–6 h, prior to challenge with 25 miracidia, only 1 (33 °C × 2 weeks), became infected, versus 36 out of 44 susceptible M-line controls (Table 1). Among the 36 infected M-line snails, 35 showed obvious daughter sporocysts in the digestive gland when removed from the shell and examined with a dissecting microscope between 21 and 33 DPE (Fig. 1A). The remaining infected M-line snail showed no external sign of infection in the digestive gland, but contained motile daughter sporocysts in the head foot when examined by tissue squash at 21 DPE (Fig. 1B).

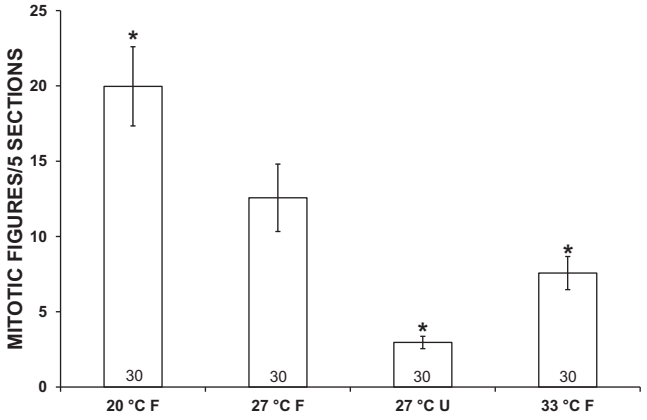

Fig. 4. Mitotic activity in the amebocyte-producing organ of *Biomphalaria glabrata* incubated for 1 week at 3 different temperatures and either fed (F) or unfed (U). Error bars indicate standard error. Numbers at bases of bars show sample size. *, mean is significantly different from 27 °C F treatment ($P < 0.05$).

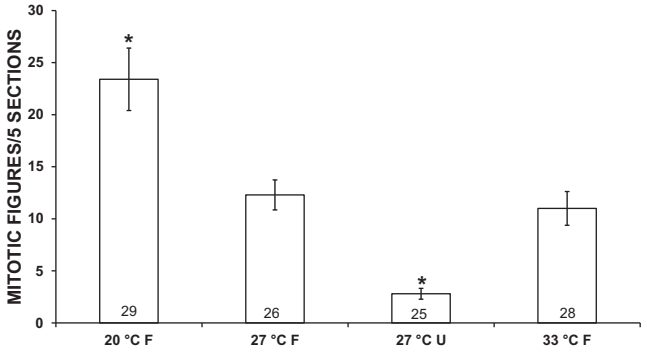

Fig. 5. Mitotic activity in the amebocyte-producing organ of *Biomphalaria glabrata* incubated for 2 weeks at 3 different temperatures and either fed (F) or unfed (U). Error bars indicate standard error. Numbers at bases of bars show sample size. *, mean is significantly different from 27 °C F treatment ($P < 0.05$).

Table 1
Prevalence of infection in adult *Biomphalaria glabrata* incubated at 3 different temperatures, either fed (F) or unfed (U), and then exposed to 25 miracidia each of *Schistosoma mansoni*. M-line controls were removed from room-temperature aquaria prior to exposure to miracidia.

Strain	Pre-exposure treatment	No. survivors/ no. exposed	No. infected
M-Line	Control	44/45	36 (82%)
Salvador	27 °C × 1 week (F)	35/37	0
Salvador	27 °C × 2 weeks (F)	34/37	0
Salvador	27 °C × 1 week (U)	35/37	0
Salvador	27 °C × 2 weeks (U)	35/37	0
Salvador	20 °C × 1 week (F)	37/37	0
Salvador	20 °C × 2 weeks (F)	33/37	0
Salvador	33 °C × 1 week (F)	32/37	0
Salvador	33 °C × 2 weeks (F)	36/37	1 (3%)
Salvador	33 °C × 5–6 h (F)	40/40	0

3.4. Hsp expression

Hsp 70 and Hsp 90 were upregulated approximately 95-fold and 5-fold, respectively, in adult Salvador snails exposed to 33 °C for 4–5 h, relative to control expression (Fig. 6). However, after 2 weeks of exposure to 33 °C, the expression levels of both Hsp 70 and Hsp 90 were below those of controls, although myoglobin expression was unaltered.

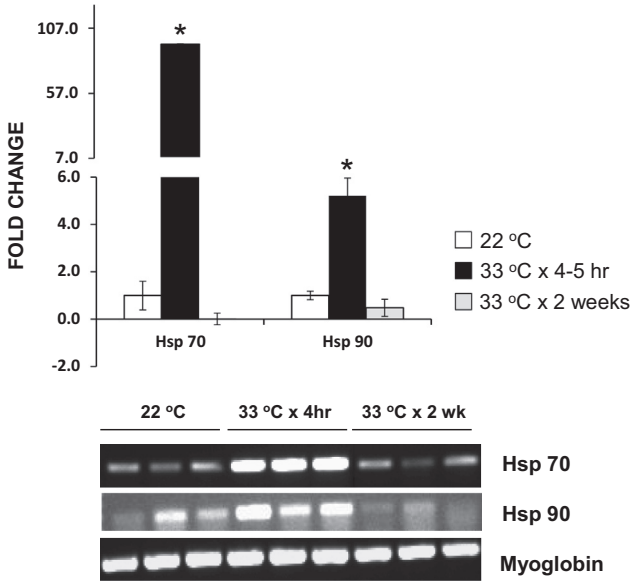

Fig. 6. Expression of Hsp 70 and Hsp 90 in *Biomphalaria glabrata* incubated at 33 °C for 4–5 h or 2 weeks, versus snails removed from room temperature (22 °C) aquaria (controls). Graph depicts mean fold change in expression in 3 snails at each treatment, each snail sample run in triplicate. *, mean differs significantly from that of controls ($P < 0.05$). Error bars indicate standard deviation. Gel photograph shows results of RT-PCR amplification of Hsp 70, Hsp 90, and myoglobin cDNA in the 3 snails from each treatment category.

4. Discussion

The results of this study demonstrate that future environmental changes may affect the IDS of *B. glabrata*. Specifically, whereas supra- and suboptimal temperatures had no effect on hemocyte concentrations, unexpectedly starvation for 1 or 2 weeks caused significant leukocytosis. The mechanism for this increase in hemocyte numbers, as well as its adaptive significance, are unclear. Higher numbers of circulating hemocytes could be due to release of hemocytes from tissue depots, prolonged hemocyte lifespan, or increased hemopoiesis. The latter possibility seems unlikely, since starvation significantly depresses mitotic activity in the APO at both 1 and 2 weeks, although hemocyte production could be occurring at other sites (see Pila et al., 2016b). Inasmuch as increased numbers of hemocytes are associated with increased resistance to schistosome infection in the 13-16-R1 strain of *B. glabrata* (Larson et al., 2014), it would be interesting to test the effects of starvation-induced leukocytosis on resistance to infection in susceptible snails.

Exposure to 20 °C for 1 or 2 weeks resulted in a significant increase in the number of mitotic figures in the APO, and yet hemocyte numbers did not increase. This effect could be a result of shorter life span of individual hemocytes or their sequestration in the tissues at the lower temperature. Additionally, rather than an increased production of hemocytes, the increased number of mitotic figures may represent a cold-induced slowdown of the mitotic phase of the cell cycle, with a resulting accumulation of cells progressing through nuclear division. However, we did not observe any obvious increase in the proportion of cells in a particular phase of mitosis, e.g., as occurs in colchicine-treated *B. glabrata* (Sullivan and Castro, 2005). The lower mitotic activity at 1 week, but not at 2 weeks, in snails exposed to 33 °C may be due to initial stress-related inhibition of cell division followed by eventual acclimation to the elevated temperature.

Results show no effect of treatments with abnormal temperatures or starvation on resistance to infection with *S. mansoni*. In particular, adult snails subjected to 33 °C for 5–6 h, 1 week, or 2 weeks remained uninfected following challenge with miracidia. In that respect, our results differ from those of Ittiprasert and Knight (2012) and Knight et al. (2015). It is possible that the method we used to assess infection, i.e., examination of the digestive gland or tissue squashes for daughter sporocysts beginning at 21 DPE, instead of cercarial emergence beginning at 4 weeks PE, as measured by Ittiprasert and Knight (2012), underestimated infection prevalences. However, while some immature infections may have been missed, infection was clearly observed in 82% of M-line controls assessed by this method (Fig. 1), and only 1 of 36 M-line snails had a cryptic infection that could not readily be discerned with a dissecting microscope. Also, by necessity we used day-old rather than fresh livers as the source of parasites, perhaps with diminished viability of miracidia. However, 82% of M-line control snails became infected, indicating adequate infectivity at a dose of 25 miracidia. Another consideration is that we used adults rather than juveniles, and as discussed by Lewis et al. (2001), mechanisms of resistance can differ in the 2 age categories of snails. Consequently, effects of heat shock may also differ. Finally, the same strain of *B. glabrata* from different laboratories can show considerable genetic heterogeneity (Mulvey and Bandoni, 1994), and the isolate of snail used in our study (originally obtained from Dr. Eric S. Loker, University of New Mexico) has been maintained independently from that in other laboratories since 1999, allowing ample time for genetic divergence. Although our method of assessing infection did not show any effect of heat shock on resistance, a histological study of the fate of sporocysts in heat-shocked and non heat-shocked snails may reveal more subtle differences between them.

It is noted that snails were maintained under the various environmental conditions in the dark. While effects of constant darkness on parameters of the IDS prior to miracidial exposure are not known, Steinauer and Bonner (2012) investigated the effect of lighting conditions during the prepatent period, and found no difference between infection prevalence in snails maintained in the dark and those exposed to a 12 h cycle of light and dark, although snails kept under dim light conditions showed a lower prevalence.

Using adult specimens, we were able to confirm prior reports of increased expression of Hsp 70 and Hsp 90 in juvenile snails following a brief exposure to high temperature. Interestingly, by 2 weeks of exposure to 33 °C the expression level of neither protein was elevated, possibly indicating that snails had acclimated to the abnormal temperature.

In conclusion, results of our study indicate that abnormal environmental conditions can have both stimulatory and inhibitory effects on hemocyte numbers and APO mitotic activity. Furthermore, some degree of acclimation to abnormal temperatures may occur. However, although elevated expression levels of heat shock proteins were seen following a 4–5 h exposure to 33 °C, none of the experimental treatments had any effect on resistance to infection with *S. mansoni*.

Acknowledgments

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Antibodies to human IL-10 neutralize ebvIL-10-mediated cytokine suppression but have no effect on cmvIL-10 activity

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ABSTRACT

Interleukin-10 is a pivotal determinant of virus clearance or persistence. Two human herpesviruses, Epstein–Barr virus (EBV) and human cytomegalovirus (HCMV) are unique among persistent viruses because they not only trigger production of host IL-10, but both viruses also encode homologs of IL-10 that are expressed during infection. Because anti-human IL-10 antibodies have diagnostic value and therapeutic potential for many chronic infections, cross-reactivity with ebvIL-10 and cmvIL-10 was evaluated in this study. Six of seven anti-hIL-10 antibodies tested recognized ebvIL-10 and neutralized its immunosuppressive activity. In contrast, cmvIL-10 was neither recognized nor neutralized by any anti-human IL-10 antibody. These findings demonstrate that IL-10-neutralizing treatments in HCMV- or EBV-infected patients may require consideration of the contribution of viral IL-10 to disease pathology.

Persistent virus infections can cause devastating human diseases. Viruses that induce chronic infections employ a range of strategies for evading the host immune system, but one mechanism has emerged as a common theme: induction of IL-10 (Blackburn and Wherry, 2007; Kane and Golovkina, 2010). IL-10 is a pleiotropic cytokine that can effectively attenuate immune responses through the suppression of inflammatory cytokines and inhibition of T cell proliferation (Mosser and Zhang, 2008). Elevated levels of IL-10 have been noted in serum from patients infected with a number of persistent viruses, including HIV, hepatitis C virus, Epstein–Barr virus (EBV), and human cytomegalovirus (HCMV) (Budiani et al., 2002; Clerici et al., 1996; Nordoy et al., 2000; Reiser et al., 1997). In addition to causing elevation of host IL-10 levels, EBV and HCMV also encode viral homologs of IL-10 that are expressed during infection.

EBV and HCMV are members of the *Herpesviridae* family. These viruses each have a large, linear DNA genome surrounded by an icosahedral capsid, an amorphous tegument layer, and an envelope containing glycoprotein spikes. In addition to a common structure, herpesviruses have the ability to establish lifelong latency in the host. Successful coexistence with the host is mediated by numerous viral gene products that modify host immune responses and create a favorable environment for virus persistence. The specific immunomodulatory viral gene products vary among the herpesviruses, and EBV and HCMV are the only human herpesviruses that encode a viral homolog of IL-10. The BCRF1 gene of EBV is expressed during the lytic cycle; the 17 kDa protein product shares 90% amino acid sequence identity with human IL-10 (hIL-10) and displays immune suppressive function (Hsu et al., 1990; Liu et al., 1997). The HCMV UL111A gene contains two introns and encodes a 17.6 kDa protein with 27% amino acid sequence identity to hIL-10 that is expressed during productive infection (Kotenko et al., 2000; Lockridge et al., 2000). Despite low sequence conservation, cmvIL-10 forms biologically active dimers with structural similarity to hIL-10, binds with high affinity to the cellular IL-10 receptor, and exhibits potent immune suppressive activity (Jones et al., 2002; Spencer et al., 2002). Alternative splicing of the UL111A gene occurs during latency, and the resulting protein, LAcmvIL-10, exhibits only a subset of immunosuppressive functions (Jenkins et al., 2004, 2008).

Elevated serum IL-10 levels generally correlate with poor prognosis in patients with chronic infections and several types of cancer (Asadullah et al., 2003; Budiani et al., 2002; Ordemann et al., 2002). In murine models, persistent lymphocytic choriomeningitis virus (LCMV) infection was found to correlate with increased IL-10 production and impaired T cell responses (Brooks et al., 2006, 2008). Neutralizing IL-10 activity with antibodies resulted in rapid virus clearance, which was also observed when IL-10 knockout mice were infected with persistent LCMV strains. Induction of IL-10 was also documented in mice infected with murine cytomegalovirus (Redpath et al., 1999), and blocking the IL-10 receptor resulted in greatly reduced viral loads (Humphreys et al., 2007). While the use of IL-10-neutralizing antibodies for the treatment of human virus infection holds promise (Ejrnaes and von Herrath, 2007; Martinic

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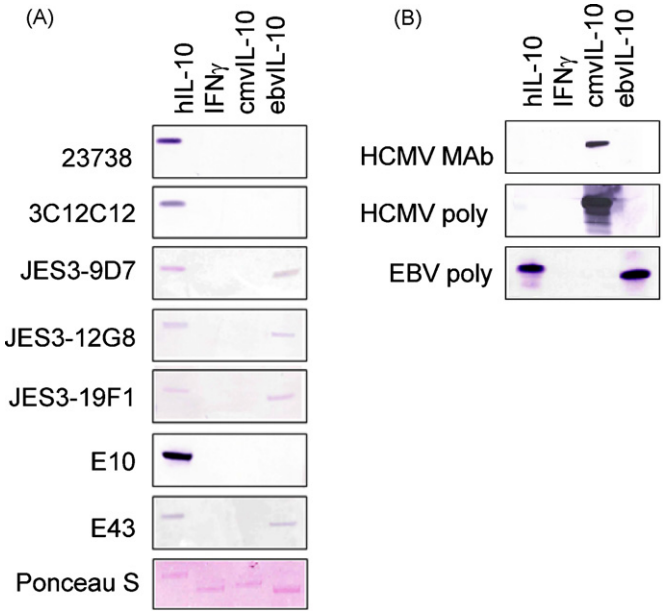

Fig. 1. Cross-reactivity of anti-hIL-10 antibodies by Western blot. Purified recombinant hIL-10, IFNγ, cmvIL-10, or ebvIL-10 (R&D Systems) were diluted to 10 μg/ml in PBS and analyzed by SDS-PAGE, transferred to a PVDF membrane, blocked, and then probed with the indicated antibodies at a dilution of 1:100 in PBS plus 5% milk. All anti-hIL-10 monoclonal antibodies are referred to by the clone name and were obtained from Santa Cruz Biotechnology. HCMV and EBV goat polyclonal antisera were obtained from R&D Systems and the HCMV monoclonal antibody was kindly provided by Dr. Gavin Wilkinson. Appropriate alkaline phosphatase conjugated secondary antibodies were used for detection at a dilution of 1:1000. Promega Western Blue Substrate was used for colorimetric detection of bands. Ponceau S staining was performed to confirm the presence of each cytokine on the membrane prior to Western blotting. Results are representative of three separate experiments.

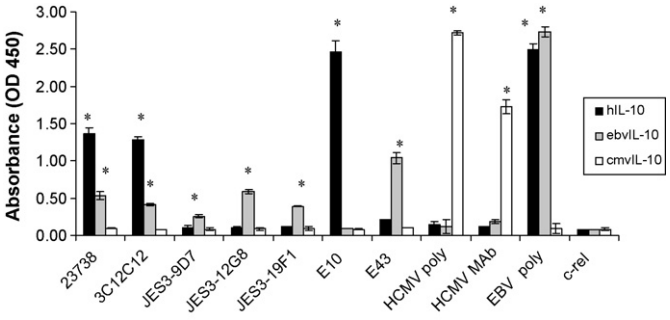

Fig. 2. ELISA detection by anti-human IL-10 antibodies. Purified hIL-10, cmvIL-10, or ebvIL-10 proteins were diluted to 2.5 μg/ml in PBS and used to coat a microtiter plate overnight at 4 °C. After blocking, the indicated antibodies were added (4 μg/ml), followed by horseradish peroxidase-conjugated secondary antibody (1:1000) and substrate reagent. An antibody to NF-κB family protein c-rel served as a negative control. Optical density was read at 450 nm following the addition of 1 M sulfuric acid stop solution to each well. Error bars represent standard error. Statistical analysis was performed using a two-tailed Student's *t*-test, and * indicates $P < 0.01$. Results are representative of four separate experiments.

and von Herrath, 2008), the existence of viral IL-10 homologs encoded by highly ubiquitous viruses like EBV and HCMV presents a serious complication in the development of treatments designed to counteract IL-10 activity.

In order to examine whether hIL-10-specific antibodies recognize cmvIL-10 or ebvIL-10, Western blots were performed. Purified recombinant human and viral IL-10 proteins were analyzed by SDS-PAGE and transferred to PVDF membranes. After blocking in PBS-Tween + 5% milk, the membranes were probed with the indicated anti-hIL-10 antibodies, followed by AP-conjugated secondary antibody. As shown in Fig. 1, all of the antibodies tested recognized hIL-10 in a Western blot. The intensity of bands varied significantly among these antibodies, with E10 consistently giving the darkest band and JES3-19F1 and JES3-12G8 showing fainter bands. All of the cytokines were present in equal amounts, as evidenced by Ponceau staining of the membranes prior to Western blotting. Interferon-gamma (IFNγ) served as a negative control cytokine and none of the antibodies showed any reaction with this protein.

Of the seven antibodies that recognized hIL-10, four of these (JES3-9D7, JES3-12G8, JES3-19F1, and E43) also detected ebvIL-10. This result is not surprising given the considerable sequence identity between hIL-10 and ebvIL-10. In contrast, cmvIL-10 has lower amino acid sequence identity with hIL-10 and was not recognized by any of the antibodies. Polyclonal antiserum directed against cmvIL-10 or ebvIL-10 was used as a control to confirm the identity of the viral cytokines. EbvIL-10 antiserum recognized both ebvIL-10 and hIL-10, while the cmvIL-10 antiserum was highly specific and did not cross-react with either hIL-10 or ebvIL-10. In addition, a monoclonal antibody raised against cmvIL-10 exhibited specificity for cmvIL-10 and failed to recognize either ebvIL-10 or hIL-10.

To evaluate antibody recognition of native protein and also allow for a relative measurement of affinity of the antibody for the target, ELISAs were performed. Purified recombinant human and viral IL-10 proteins were adsorbed to a microtiter plate for 15 h at 4 °C. The plate was washed, blocked with PBS containing 1% BSA, and then anti-hIL-10 antibodies were added at a concentration of 2 μg/ml. After incubation for 2 h at room temperature, the plates were washed and antibody binding detected via the addition of HRP-conjugated secondary antibody. As shown in Fig. 2, three of the seven antibodies recognized hIL-10 in this assay. Antibody clones 23738, 3C12C12, and E10 all had absorbance values that were significantly higher than the background readings from the negative control c-rel antibody (Santa Cruz Biotechnology). Unexpectedly, four of the anti-hIL-10 antibodies that recognized hIL-10 by Western blot did not react with hIL-10 via ELISA (JES3-9D7, JES3-12G8, JES3-19F1, and E43). This suggests that either the epitopes for these antibodies may require protein denaturation or that adsorption to the ELISA dish obscured the epitope. Despite these possible limitations, all four of these antibodies recognized ebvIL-10 by both ELISA and Western blot. Again, none of the anti-hIL-10 antibodies recognized cmvIL-10, and the cmvIL-10 antibodies reacted with cmvIL-10 only.

To further examine the specificity of these antibodies, a neutralization assay was performed. Suppression of inflammatory cytokines is a hallmark property of human and viral IL-10s (Moore et al., 2001; Slobedman et al., 2009), and hIL-10 was initially referred to as CSIF, or cytokine synthesis inhibitory factor (Fiorentino et al., 1989). The ability of each antibody to reduce IL-10-mediated cytokine suppression was evaluated. THP-1 monocytes were treated with 1 ng/ml lipopolysaccharide (LPS) to stimulate production of TNFα. In the presence of hIL-10, cmvIL-10, or ebvIL-10, TNFα production is greatly reduced, as described previously (Spencer, 2007; Spencer et al., 2002). Treatment with anti-hIL-10 antibodies neutralized CSIF activity and in several cases, fully restored TNFα levels (Fig. 3). An antibody to the human cellular IL-10 receptor (R&D Systems AF874) served as a positive control for neutralization and was effective at inhibiting cytokine suppression by hIL-10, cmvIL-10, and ebvIL-10. Six of the anti-hIL-10 antibodies almost completely neutralized both hIL-10 and ebvIL-10 CSIF activity, but no effect on cmvIL-10 function was observed with any anti-hIL-10 antibody. The ebvIL-10 antiserum was effective against both hIL-10 and ebvIL-10. Only the anti-cmvIL-10 antibodies neutralized cmvIL-10-mediated immune suppression, and these antibodies had no effect on the CSIF activity of either hIL-10 or ebvIL-10.

Antibody E10 exhibited the greatest specificity for hIL-10 but was unable to fully neutralize cytokine suppression, suggesting that

Table 1
Cross-reactivity of IL-10-specific antibodies.

Clone	Isotype	Commercial source(s)	Reactivity		
			hIL-10	ebvIL-10	cmvIL-10
23738	Mouse IgG2b	Abcam, Genway, Leinco Technologies, R&D Systems, Santa Cruz Biotech, Sigma–Aldrich	+[a]	+[b]	–
3C12C12	Mouse IgG1	CytoMol UniMed, LifeSpan Biosciences, Novus Biologicals, Santa Cruz Biotech	+[a]	+[b]	–
JES3-9D7	Rat IgG1	Abcam, AbD Serotec, BD Pharmingen, Biolegend, Beckman Coulter, Biolegend, eBioscience, GeneTex, Inc., Genway, LifeSpan Biosciences, Novus Biologicals, Santa Cruz Biotechnology, Southern Biotech, Thermo Scientific	+[c]	+[a]	–
JES3-12G8	Rat IgG2a	AbD Serotec, BD Pharmingen, Beckman Coulter, Biolegend, eBioscience, LifeSpan Biosciences, Novus Biologicals, Santa Cruz Biotechnology, Southern Biotech, Thermo Scientific	+[c]	+[a]	–
JES3-19F1	Rat IgG2a	BD Pharmingen, Biolegend, Santa Cruz Biotechnology	+[c]	+[a]	–
E10	Mouse IgG2b	Santa Cruz Biotechnology	+[d]	–	–
E43	Mouse IgG2b	Santa Cruz Biotechnology	+[c]	+[a]	–
EBV poly	Goat IgG polyclonal	R&D Systems (Catalog # AF915)	+[a]	+[a]	–
HCMV poly	Goat IgG polyclonal	R&D Systems (Catalog # AF117)	–	–	+[a]

[a] Western blot, ELISA, and neutralization.
[b] ELISA and neutralization only.
[c] Western blot and neutralization only.
[d] Western blot and ELISA only.

the epitope for E10 may be distinct from the receptor contact sites. While E10 showed no recognition of ebvIL-10 via Western blot or ELISA, a modest decrease in CSIF activity was observed when this antibody was included in the reaction mix. EbvIL-10 has been shown to have greatly reduced affinity for the IL-10 receptor compared with hIL-10 (Liu et al., 1997), which may also account for the near complete neutralization of ebvIL-10 activity by antibodies that exhibited relatively weak recognition by Western blot or ELISA. The results for all of the commercially available antibodies tested are summarized in Table 1.

IL-10 is an important regulator of anti-inflammatory activity in infection, autoimmune diseases, and cancer. The existence of viral IL-10s produced by herpesviruses that are widespread in the general population complicates both the measurement of IL-10 levels and therapeutic efforts to ameliorate IL-10 activity. Our results show that many anti-hIL-10 antibodies can recognize and neutralize both hIL-10 and ebvIL-10, but these antibodies do not detect cmvIL-10 or neutralize its biological activity. Due to the lack of recognition of cmvIL-10, LAcmvIL-10 was not tested for cross-reactivity with these anti-hIL-10 antibodies. LAcmvIL-10 is a truncated protein of 139 amino acids that is co-linear with cmvIL-10 for the first 127 amino acids (Jenkins et al., 2004). The C-terminal 12 amino acids of LAcmvIL-10 have no significant sequence similarity to either cmvIL-10 or hIL-10 (data not shown); thus, it is highly unlikely that hIL-10-specific antibodies that fail to recognize full length cmvIL-10 would detect LAcmvIL-10.

Detection of ebvIL-10 by some IL-10 reagents has previously been noted (Blay et al., 1993; de Waal Malefyt et al., 1991); however, this study represents the most comprehensive comparison of antibody cross-reactivity between hIL-10, ebvIL-10, and cmvIL-10. Although the antibodies tested here are marketed for research purposes, they could be produced for use as diagnostic tools or therapeutic treatments in the future. One antibody, JESG-12G8, has already been humanized and has potential for use as a clinical treatment (Presta, 2005). Given the success of other humanized monoclonal antibody therapeutics, anti-IL-10 antibodies could soon become important clinical tools, making it imperative that cross-reactivity with viral IL-10 homologs be thoroughly examined.

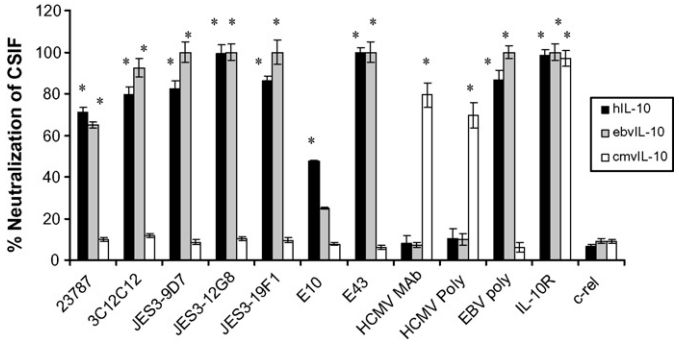

Fig. 3. Effect of anti-human IL-10 antibodies on TNFα production. THP-1 monocytes were seeded into 96-well culture dishes at 2×10^4 cells per well and treated with 1 ng/ml LPS in the presence or absence of hIL-10, cmvIL-10, or ebvIL-10 (10 ng/ml). After 24 h, supernatants were collected and TNFα levels determined by ELISA (R&D Systems) to determine total cytokine suppression for each cytokine. The indicated antibodies (5 μg/ml) were included just prior to the addition of human or viral IL-10 to neutralize CSIF activity. Results are expressed as percent neutralization, or the extent to which antibodies relieved TNFα suppression caused by the cytokines alone. Error bars represent standard error. Statistical analysis was performed using a two-tailed Student's *t*-test, and * indicates $P < 0.01$. Results are representative of four separate experiments.

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Research Article Open Access

Synthesis and Evaluation of Ornithine Decarboxylase Inhibitors with Oxime Moiety and MCF-7 Breast Cancer Cells

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Abstract

Cell proliferation can be regulated by small, aliphatic polyamines, and it is suggested that tumor tissues have significantly higher polyamine levels than surrounding tissues. The major biologically active polyamines present in mammalian cells are putrescine, spermidine, and spermine. The Ornithine Decarboxylase (ODC) catalyzes the decarboxylation of ornithine to produce putrescine which is aprecursor of polyamine synthesis. We report here the synthesis of 2-Amino-5-(Hydroxyimino) Pentanoic Acid (AHPA), based on the substrate of ODC, L-ornithine, derivatized with oxime functionality. In molecular docking studies, the E-isomer AHPA binds to ODC more favorably than does the *Z*-isomer. In addition, the growth of MCF-7 (Michigan Cancer Foundation–7) breast cancer cells in the presence of AHPA was significantly reduced. These results implicate that AHPA can be explored as a potential agent of cancer chemotherapy.

Keywords: Polyamine; Ornithine decarboxylase (ODC); Inhibitors of ODC; MCF-7 (Michigan Cancer Foundation–7)

Introduction

Cancer is the second most common cause of death for Americans and accounts for nearly 1 of every 4 deaths in the US [1,2]. An estimated 229,060 new cases of invasive breast cancer are expected to occur among women in the US during 2012; about 2,190 new cases are expected in men [1,2]. Cancer is a disease related to uncontrolled growth and spread of abnormal cells [2]. Targeting the polyamine pathway has been studied in possible therapeutic approaches [3]. One of the most promising areas for the development of novel anti-cancer therapeutics is polyamine biosynthesis [4,5]. The finding that inhibitors of polyamine biosynthesis can prevent, or at least limit cancer cell growth [6-9], together with the fact that polyamine concentrations are elevated in multiple cancer tissues [10-12], has made polyamine metabolism a promising target for cancer chemoprevention and therapy. The major biologically active polyamines present in mammalian cells are putrescine, spermidine, and spermine [3]. These molecules are synthesized in sequence starting from ornithine, which is derived from the amino acid arginine through the action of the enzyme arginase (Scheme 1). The first critical step is the synthesis of putrescine via the decarboxylation of ornithine, which is catalyzed by the enzyme Ornithine Decarboxylase (ODC) [13,14]. Subsequent steps involve the production of spermidine through the addition of Decarboxylated S-Adenyosylmethionine (DAM) to the putrescine by spermidine synthase. A second DAM is then added to spermidine to produce spermine [15-17] (Scheme 1).

Difluoromethylornithine (DFMO), an inhibitor of the first enzyme in the mammalian polyamine biosynthetic pathway, ornithine decarboxylase, is approved for use in trypanosomiasis and has shown promise in the therapy of brain tumors [18]. DFMO was originally evaluated as an antitumor agent in the early 1980s, with limited success. Phase I studies suggested a dose of 2.25 g/m^2 every 6 h for patients with advanced solid tumors or lymphomas [19]. Phase II studies were carried out with melanoma patients including small cell lung carcinoma, colon cancer, and prostate cancer [20-22]. The drug was generally well tolerated, although significant but infrequent adverse effects including thrombocytopenia (a relative decrease of platelets in blood), transient hearing loss, and osmotic diarrhea were noted. The results of these studies deterred continued evaluation of the drug as an antitumor agent. Although the ODC inhibitor may have significant effects on their respective target enzymes, only one inhibitor, *R*-Difluoromethylornithine (DFMO), has reached the market. DFMO was originally designed as an antitumor agent, but the drug was not effective enough to the further study of phase II trials. However, it has been shown to be an effective cure for infection caused by *Trypanosoma brucei gambiense*, which causes West African sleeping sickness [23,24].

Due to the limitation of use of DFMO, it is necessary to design new possible compounds that disturb the polyamine biosynthetic pathway. The oxime moiety is of great value in investigating binding affinities in arginine biosynthesis due to the geometrical isomers (*E/Z*) of the oxime functionality and biological activities [25]. The oxime moiety (-C=N-OH) is easily coordinated with metal ions or hydrogen bonds with conserved residues in active sites of various enzymes.

In this present study, we report the design and synthesis of 2-amino-5-(hydroxyimino) pentanoic acid (AHPA), 6, which contains an oxime functional group. The molecular docking study was conducted to extend Structure-Activity Relationship (SAR) studies based on AHPA with ODC (PDB code 2ON3). Although we synthesize the mixture of E and *Z* isomers of 2-Amino-5-(Hydroxyimino) Pentanoic Acid (AHPA), we can anticipate that the E isomer has a better binding affinity than the *Z*-isomer based on a molecular docking study. In

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addition, the biological evaluation of AHPA with MCF-7 (Michigan Cancer Foundation–7) breast cancer cells was shown more potent when compared with DFMO *in vitro*. Our initial studies investigated the impact of AHPA on proliferation of human breast cancer cells, and the results indicate that inhibition of ODC greatly impairs the ability of these cells to replicate. Therefore, AHPA may have a considerable potential as a cancer chemopreventive and therapeutic agent.

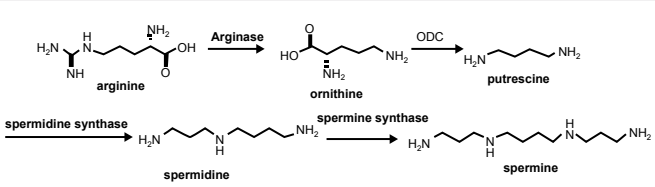
Results and Discussion

Chemistry

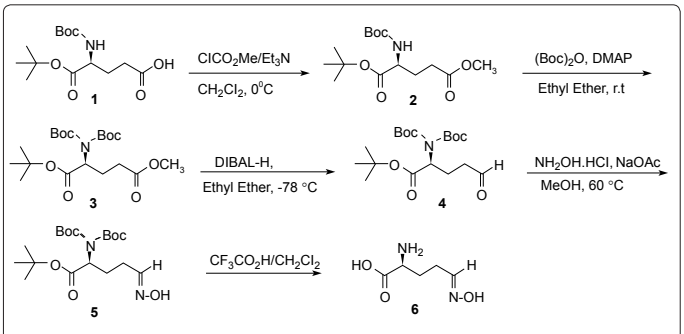
We designed and synthesized a 2-amino-5-(hydroxyimino) pentanoic acid 6 (AHPA) inhibitor based on the modified substrate of ornithine decarboxylase with oxime functionality. The Compound 6 (APHA) was synthesized using the procedure described in scheme 2.

2-Amino-5-(hydroxyimino)pentanoic acid (AHPA), 6, was synthesized using L-glutamic acid derivative *(S)*-5-(*tert*-butoxy)-4-[(*tert*-butoxycarbonyl)amino]-5-oxopentanoic acid 1, as the starting material. Esterification of the compound 1 using methyl chloroformate by treatment with triethylamine and a catalytic amount of 4-dimethylaminopyridine (DMAP) in dry methylene chloride provided the ester derivative 2. Synthesis of di-*tert*-butyl dicarbonate (di-BOC) 3 was carried out by treatment with di-*tert*-butyl dicarbonate in the presence of DMAP in methylene chloride. The subsequent reduction of ester derivative 3 was performed with diisobutylaluminium hydride (DIBAL-H) in ether to provide the aldehyde 4 [26]. Oxime derivative 5 was prepared for hydroxylamine hydrochloride in methanol under reflux condition. Complete deprotection of the compound, 5 with trifluoroacetic acid (TFA) in methlyene chloride yielded AHPA 6.

We have investigated the stabilities of geometric isomers in molecular modeling-docking studies to computationally evaluate the fit between the human and *Leishmania donovani* ODC (PDB code 2ON3) and the *E*- and *Z*-isomers of AHPA [27,28]. Calculated binding data are recorded in table 1. When *E*-AHPA is bound in an extended conformation, the oxime moiety can make hydrogen bonds with conserved enzyme residues, Lys 69, Arg154, and Glu274. When *Z*-AHPA is bound, the oxime moiety can make a hydrogen bond



Scheme 1: Biosynthesis of Polyamines.



Scheme 2: BSynthesis of 2-amino-5-(hydroxyimino)pentanoic acid (AHPA).

with only one conserved residue, Asp364. Thus, the greater hydrogen bonding potential of the *E*-isomer of the oxime moiety suggests a stability preference for this isomer. Force-field based methods can predict the binding free energy of a protein-ligand complex by adding up individual contributions from different types of interactions. Programs for energetic analysis of receptor-ligand interaction based on force-field scoring functions and terms including van der Waals, electrostatics and hydrogen bonds can be available.

Interestingly, Autodock molecular modeling of the structure of human and *Leishmania donovani* ornithine decarboxylases14 (PDB code 2ON3) with geometric isomers (*E/Z*) of oxime ligands has shown that a high degree of affinity with *E* isomer rather than *Z* isomer (Table 1, Figure 1 and Figure 2).

To evaluate the impact of ODC inhibitors on cell proliferation, MCF-7 cancer cells were used. The cells were cultured in the presence or absence of varying doses of AHPA. As shown in figure 3, AHPA treatment significantly reduced cell proliferation. A seven point dilution series was performed, and even at the lowest concentration (0.391 mg/ml), AHPA was found to affect cell viability. At 24 hours post-treatment, the cells were viable and the level of ATP was roughly equivalent for cells under each experimental condition. By 48 hours post-treatment, there was no proliferation detected and viability of all cells that had been exposed to AHPA was decreasing. In contrast, the control cells exhibited healthy and robust proliferation, as evidenced by the increasing amounts of ATP detected. By 96 hours, cells that had been exposed to AHPA at any dose were dead. It thus appears that AHPA was a much more potent inhibitor of cell proliferation than comparable doses of DFMO (difluoromethylornithine), a well-known inhibitor of ornithine decarboxylase. Although there is a possibility that the reduction of cell proliferation by AHPA is due to the compound toxicity, the significant effect of AHPA on cell proliferation and the *in silico* analysis support the conclusion that the AHPA inhibit the cell proliferation by binding to ODC.

`MCF-7 breast cancer cells were cultured in the presence of absence of AHPA at the indicated time points. Cell viability and proliferation was measured via the Cell Titer Glo Assay, and results are expressed as relative light units.

As shown in figure 4, MCF-7 cell proliferation was not significantly affected by doses of DFMO lower than 3 mg/ml. At doses higher than 3 mg/ml DFMO, modest reduction of cell proliferation was observed. Proliferation of cells treated with AHPA was dramatically reduced by even the lowest doses of the compound. These results demonstrate that AHPA is a potent inhibitor of cell proliferation.

In conclusion, while exploring the design of new therapeutic inhibitors in arginine biosynthetic pathways, AHPA appears to be a potential cancer chemotherapeutic agent based on the observations of *in silico* docking and cell proliferation experiments. The docking studies have shown that geometric isomers of the ornithine-based

Compound AHPA, 6	N_{tor}	ΔG_{AD4} (kcalmol)	RMSD (Å)	H-bonding interaction	H-bond distance (Å)
E-isomer *(trans)*	3	-7.92	2.347	HO…HN(Lys 69) HO…HN(Arg154) NH…OH(Glu274)	2.7 1.9 1.8
Z-isomer *(cis-)*	6	-5.51	0.485	OH…OC(Asp364)	1.9

Table 1: Comparison of the complex of *E/Z* isomers of 2-Amino-5-(Hydroxyimino) Pentanoic Acid (AHPA) with ODC (PDB code 2ON3) in molecular docking studies

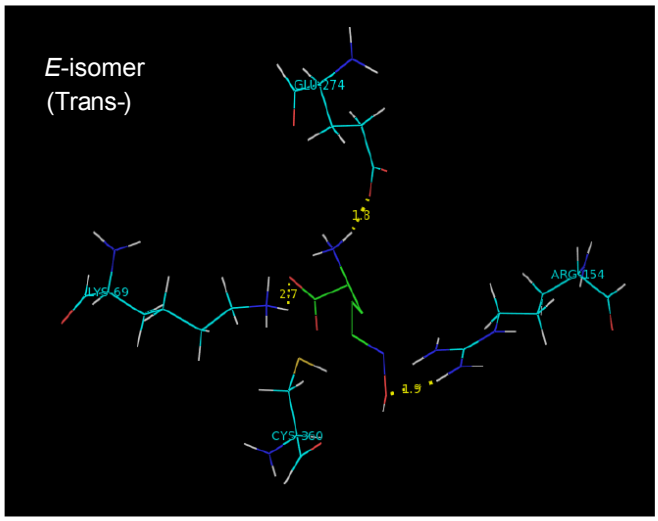


Figure 1: A schematic diagram showing the interactions of *E*-AHPA with neighboring groups within the active site of ODC (PDB code 2ON3)

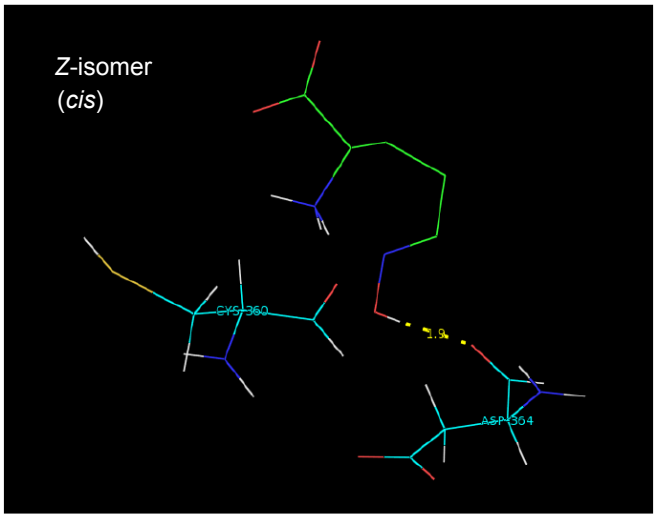


Figure 2: A schematic diagram showing the interactions of Z-AHPA with neighboring groups within the active site of ODC (PDB code 2ON3)

oxime AHPA play a significant biological role in blocking ODC activity. The predicted binding affinity of the *E*-isomer of AHPA is higher than that of its *Z*-isomer with ODC as calculated with AutoDock. Besides, AHPA significantly affects cell viability with MCF-7. It is, however, worth noting that the *in silico* and cell proliferation observations will not warrant the fact that the AHPA specifically binds to ODC. To assess this issue, more evidence by further investigations would conclusively support the binding specificity. Furthermore, exploring structure-activity relationships of additional AHPA analogues as well as determining their lowest effective doses would be considered as future studies.

Experimental section

Synthesis of an amino acid oxime analogue: Reactions requiring anhydrous conditions were carried out under a nitrogen atmosphere in oven-dried glassware, and solvents were freshly distilled. Diethyl ether and Tetrahydrofuran (THF) were distilled from sodium/benzophenone. Dichloromethane and triethylamine were distilled from calcium hydride. All other reagents and solvents were used without further purification from commercial sources. Organic extracts were dried over anhydrous $MgSO_4$ or Na_2SO_4. Reactions were monitored by Thin-Layer Chromatography (TLC) with 0.25-mm E. Merck precoated silica gel plates and visualized with ninhydrin solution (0.1% ninhydrin in 95% *n*-butanol, 4.5% water, 0.5% glacial acetic acid) and $KMnO_4$ solution (3 g of $KMnO_4$, 20 g of K_2CO_3, 5 ml of 5% NaOH, and 300 ml of water). Flash column chromatography was carried out with E. Merck silica gel 60 (230-240 mesh ASTM). The 1H and 13C NMR spectra taken by Dr. Garner by were recorded on a Bruker AM-500 spectrometer. Chemical shifts were expressed in parts per million (ppm) and referenced to CDCl3, CD3OD, or DMSO-*d6* at Baylor University. The general syntheses of *(S)-tert*-butyl 2- (bis(*tert*-butoxycarbonyl)amino)-5-(hydroxyimino)pentanoate 5 and *(S)*-2-amino-5-(hydroxyimino) pentanoic acid (AHPA) 6 are as follows.

Procedure

(S)-tert-butyl 2-(bis(*tert*-butoxycarbonyl)amino)-5-(hydroxyimino) pentanoate, 5

At room temperature to a solution of the appropriate aldehyde (1.0 g, 8.9 mmol) in methanol (5 ml) was added hydroxyamine hydrochloride (0.743 g, 0.107 mmol) followed by sodium acetate trihydrate (0.148 g,

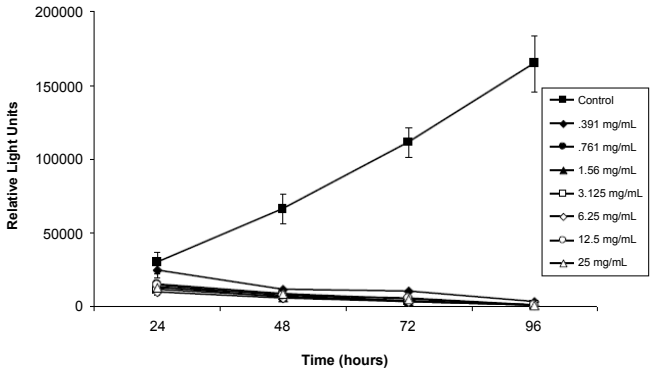


Figure 3: Effect of AHPA on cell proliferation.

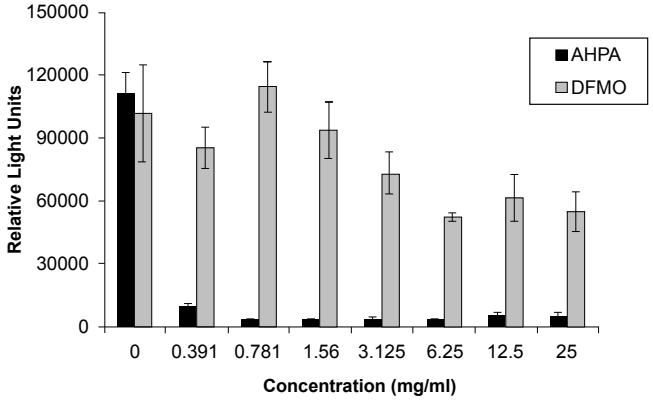


Figure 4: MCF-7 cells were cultured in the presence of DFMO or AHPA at the indicated concentrations and cell viability was measured at 72 hours using the Cell Titer Glo Assay. Results are representative of two separate experiments and error bars represent standard error

0.107 mmol) and methanol (10 ml). Enough methanol was then added to give a clear solution, and stirring at 60°C was refluxed for 3-4 hrs. The resulting solution was cooled to room temperature, diluted with water (10 ml), and extracted with ether (3×10 ml). The combined organic phases were washed with saturated sodium bicarbonate (2×10 ml) and brine and then dried over $MgSO_4$. The solvent was removed at reduced pressure, and the crude oximes was purified by column chromatography (1:9=ethylacetate:hexanes): clear oil. Yield (0.5 g 81.45%).

^{1}H NMR (CDCl3): δ 7.49-7.41 (t, 0.56H), 6.83-6.73 (s, 0.47H), 4.85-4.73 (m, 1H), 2.15-1.99 (m, 1H), 1.54-1.40 (m, 29H). IR (cm-1): 3550-2800, 1800-1700. MS (ES+) *(m/z)*: [M+Na] calcd for ($C_{19}H_{34}N_2O_7Na$) 425.2264, found 424.2245.

2-amino-5-(hydroxyimino)pentanoic acid (AHPA), 6: To a stirred solution of the compound 5 (0.425 g, 1.03 mmol) in CH_2Cl_2 (10 ml) was slowly added Trifluoroacetic Acid (TFA) (8 ml) at 0°C. The reaction mixture was stirred for 1-4 hrs and then allowed to equilibrate at room temperature until the end of the reaction. The solvent was removed on a rotary evaporator, and the crude residue was purified by silica gel chromatography to yield 6 (0.24 g, 87% yield).

^{1}H NMR (MeOD): δ 7.45-7.33 (m, 0.39H), 6.78-6.68(m, 0.29H), 4.08-3.90 (m, 1H), 2.25-1.95(m, 2H), δ1.38-1.20(m, 0.72H). IR (cm^{-1}): 3500-2100, 1800-1600.

MS (ES+) *(m/z)*: [M+H] calcd for ($C_5H_{11}O_3N_2$) 147.0770, found 147.0764.

Preparation of Target Protein Structure

The 3D structure of ODC (Ornithine Decarboxylase) from human and *Leishmania donovani* was obtained from the protein data bank (PDB code 2ON3). The X-ray crystal structure of the enzyme has been determined at 3.0 Å resolution [14]. All atom parameters were automatically created by AutoDockTools (ADT). Coordinates for the ODC (PDB code 2ON3) were processed in ADT by adding hydrogen atoms, assigning charges with the Gasteiger method, and merging the non-polar hydrogens. The 3D structure and active site residues of target protein were confirmed by PyMol [27].

Docking with Predefined Compounds

To increase the validity of potential inhibitor and docking studies, inhibitor candidates (geometric isomers (*E/Z*) of oxime ligands) were chosen as control dockings. We utilized the Dundee PRODRG28 server to generate a topological description of the both *E* and *Z* form. This server converted 2D compounds drawn by the JME editor to 3D coordinates in PDB format, adding hydrogen atoms. The 3D structures of the compounds, in the PDB format, were then docked with the (PDB code 2ON3) using AutoDock4.0 (http://autodock.scripps.edu). A grid map of 60Å×60Å×60Å points with 0.375Å grid spacing was centered on the active site and was calculated around the docking area by running AutoGrid. The Lamarckian genetic algorithm was used as the search method, and the docking parameters were set to 10 automated docking runs, as default setup, for a 150 population size with a 2,500,000 maximum number of energy evaluations for each docking experiment. The results showed the binding energies and hydrogen bonds' interactions between the ligands and (PDB code 2ON3), and the cluster analysis was performed based on Root Mean Square Deviation (RMSD).

Biological Assays

MCF-7 breast cancer cells were maintained in FK12/DMEM medium supplemented with 5% fetal bovine serum in a 37°C incubator with a humidified 5% CO_2 atmosphere. Cells were plated at 5×10^4 per well in white 96 well plates in the presence or absence of compound as indicated and each treatment was performed in triplicate. Cell growth was measured at the indicated time points using the Cell Titer Glo Kit (Promega, Madison, WI). Briefly, a luciferin substrate added to each well was converted to oxyluciferin in the presence of O_2 and ATP. The light produced was measured using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) and the relative light units were proportional to the amount of ATP present, reflecting the number of viable cells in the well. DFMO was purchased from Sigma-Aldrich (St. Loius, MO).

Acknowledgment

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Stimulation of B lymphocytes by cmvIL-10 but not LAcmvIL-10

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Abstract

Human cytomegalovirus (HCMV) is a widespread pathogen that establishes lifelong latent infection facilitated by numerous mechanisms for modulating the host immune system. The UL111A region of the HCMV genome encodes a homolog of human cellular IL-10 (hIL-10). The viral cytokine, cmvIL-10, exhibits many of the immunosuppressive properties of hIL-10. However, hIL-10 is also known to have stimulatory effects on B lymphocytes. We found that cmvIL-10 has the ability to enhance B cell proliferation, despite having only 27% sequence identity to hIL-10. Treatment with cmvIL-10 stimulated autocrine production of hIL-10 by B lymphocytes and led to activation of the latent transcription factor Stat3. In contrast, LAcmvIL-10, a truncated protein resulting from an alternatively spliced transcript in latently infected cells, did not stimulate B cell proliferation, Stat3 activation, or hIL-10 production. These results provide insights into the biological activity of the full-length and latency-associated viral cytokines and suggest different roles for each in HCMV infection.

Keywords: Human cytomegalovirus; Immune evasion; Viral cytokine; Interleukin-10

Introduction

Human cytomegalovirus (HCMV) is ubiquitous in the general population, causing acute, chronic, or latent infection. Once known as "salivary gland virus", HCMV was first isolated from the salivary glands and kidneys of a dying infant with greatly enlarged cells, or cytomegalic inclusion bodies, in affected tissues (Brennan, 2001). In most individuals, primary infection with HCMV is asymptomatic; however, serious symptoms can occur in patients with compromised immune systems (Britt, 1996). The HCMV genome is among the largest of mammalian DNA viruses (>230 kb), and it encodes more than 200 different proteins (Murphy et al., 2003). A significant part of this coding potential is devoted to proteins that are not required for virus replication in culture, but instead, to proteins that facilitate immune evasion and virus persistence *in vivo*. Among these immunomodulatory proteins is the product of the UL111A region, which encodes a protein with 27% amino acid sequence identity to human interleukin-10 (hIL-10) (Kotenko et al., 2000).

IL-10 is a multi-functional cytokine that is important in the regulation of the immune response (Moore et al., 2001). Secreted by numerous cell types, including macrophages, T cells, and B cells, hIL-10 is normally produced late in the immune response to a pathogen when it functions to attenuate the response through suppression of inflammatory cytokines. Exploitation of hIL-10 and its immunomodulatory properties is a common strategy for intracellular pathogens. Bacteria like *Listeria monocytogenes* and *Mycobacterium tuberculosis* induce production of cellular hIL-10 to avoid immune clearance (Redpath et al., 2001), while numerous viruses encode homologs of hIL-10, including HCMV, Epstein–Barr virus (Hsu et al., 1990), equine herpesvirus 2 (Rode et al., 1993), and the Orf poxvirus (Fleming et al., 1997). HCMV-encoded IL-10 is unique among these viral homologs because it has significantly lower sequence identity to hIL-10, and because it is encoded as a discontinuous open reading frame containing two introns (Kotenko et al., 2000). Intriguingly, the presence of introns in the gene encoding cmvIL-10 allows for the

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possibility of alternative splicing, and this has been documented to occur in latently infected granulocyte–macrophage progenitor cells (Jenkins et al., 2004). The UL111A region latency-associated (LA) transcript differs from full-length cmvIL-10 transcripts in that it contains only one intron, resulting in an in-frame stop codon at nucleotide position 160171 (strain AD169). The LAcmvIL-10 protein product is co-linear with cmvIL-10 for the first 127 residues and then diverges in sequence at the truncated C-terminal domain (139 amino acids total compared to 175 for full-length cmvIL-10). Whereas full-length cmvIL-10 exhibits a broad range of inhibitory functions associated with hIL-10, including inhibition of PBMC proliferation, suppression of inflammatory cytokine synthesis, reduction of class II MHC expression, and impairment of dendritic cell maturation expression (Chang et al., 2004; Raftery et al., 2004; Spencer et al., 2002), the immunosuppressive activities of LAcmvIL-10 appear to be much more restricted. To date, LAcmvIL-10 has been shown only to induce down-regulation of class II MHC on myeloid cells, and this was found to occur independently of the cellular IL-10 receptor (B.S., unpublished data).

In addition to its potent anti-inflammatory properties, hIL-10 also plays a key role in promoting the growth and differentiation of B cells (Go et al., 1990; Moore et al., 2001; Rousset et al., 1992). Such stimulatory activity, however, has not yet been reported for cmvIL-10 or LAcmvIL-10. Considering their relatively low sequence identity with hIL-10, it seems likely that these viral cytokines might preserve only a distinct subset of hIL-10 activities (i.e., those that are immunosuppressive) in order to produce an environment that is advantageous for the virus. Here, we investigated whether cmvIL-10 had retained the ability to promote B cell proliferation. Our results show that cmvIL-10 stimulates both B cell growth and autocrine production of cellular hIL-10, whereas LAcmvIL-10 does not. These findings provide additional functional characterization of cmvIL-10 and highlight functional differences between the full-length and truncated HCMV IL-10 homologues.

Results

To investigate whether cmvIL-10 had retained the ability to stimulate B cell proliferation, we employed the human B cell lymphoma Daudi cell line. Daudi B cells were cultured in RPMI containing 10% fetal calf serum and supplemented with 5 ng/ml recombinant human IL-4. In control cells, significant cell growth was observed over a 72-h time period. When treated with cmvIL-10, however, cell growth was increased by approximately 45% after 48 h (Fig. 1A). By 72 h, cmvIL-10 treatment had increased proliferation by nearly 60% over the basal rate. This outcome was similar to treatment with the same dose of hIL-10, which caused B cell proliferation to increase to levels of 64% higher than control cells. Overall, B cell proliferation was enhanced in a dose-dependent manner, as shown in Fig. 1B, confirming that cmvIL-10 has comparable stimulatory activity to the human cellular cytokine. In contrast, LAcmvIL-10 treatment did not increase B cell growth by more than 5–10% over basal levels at any dose or time point, suggesting that the latent form of the viral cytokine may have distinct biological functions from the full-length cmvIL-10.

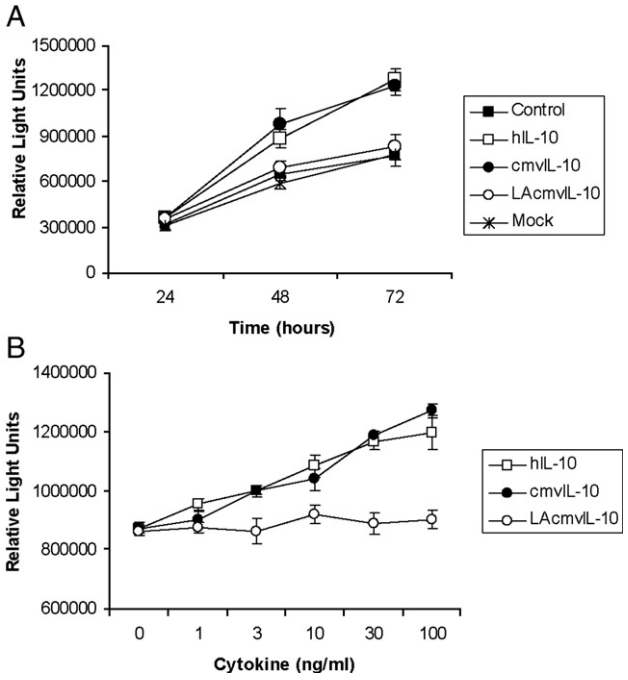

Fig. 1. cmvIL-10 stimulates proliferation of B cells. (A) Daudi B cells were cultured in control medium containing 10% FCS and 5 ng/ml rhIL-4 or medium supplemented with 30 ng/ml recombinant hIL-10, cmvIL-10 or LAcmvIL-10. Control cells received an equivalent volume of the mock protein preparation. Proliferation was measured at the indicated times using the luciferin-based Cell TiterGlo Assay (Promega); the resulting light emitted was recorded in relative light units (RLUs) and is proportional to cell number. This figure is representative of three separate experiments. (B) Dose-dependent stimulation of B cells by cmvIL-10. Daudi B cells were cultured in the presence of the indicated doses of hIL-10, cmvIL-10 or LAcmvIL-10. After 72 h, the Cell Titer Glo assay was used to quantify cell number. ($n=3$ separate experiments). Error bars indicate standard deviation from the mean value for three replicate data points.

Previous work has demonstrated that the immune suppressive effects of cmvIL-10 are mediated through engagement of the cellular IL-10 receptor (Kotenko et al., 2000; Spencer, 2007; Spencer et al., 2002). One of the earliest outcomes resulting from ligand engagement of the IL-10R is activation of the latent transcription factor Stat3 by the receptor-associated kinase JAK1 (Moore et al., 2001). To examine whether the stimulation of B cell growth by cmvIL-10 also led to activation of Stat3, Daudi B cells were treated with varying doses of hIL-10, cmvIL-10, or LAcmvIL-10 for 15 min, then cell lysates were analyzed by SDS–PAGE and Western blotting. Stat3 activation induced by cmvIL-10 was evident from the phosphorylation of tyrosine 705, as shown in Fig. 2A. Comparable results were observed with hIL-10, confirming that cmvIL-10 has retained the capacity to mimic the activation properties of hIL-10. In contrast, LAcmvIL-10 did not induce Stat3 activation in B cells. When cells were treated with both cmvIL-10 and LAcmvIL-10 in combination, Stat3 phosphorylation was observed (data not shown), confirming that LAcmvIL-10 did not actively inhibit or interfere with Stat3 activation.

Although previous studies demonstrating biological function of full-length cmvIL-10 were performed with a recombinantly expressed protein containing a 6X-histidine epitope tag (Spencer et al., 2002), our current work has employed commercially

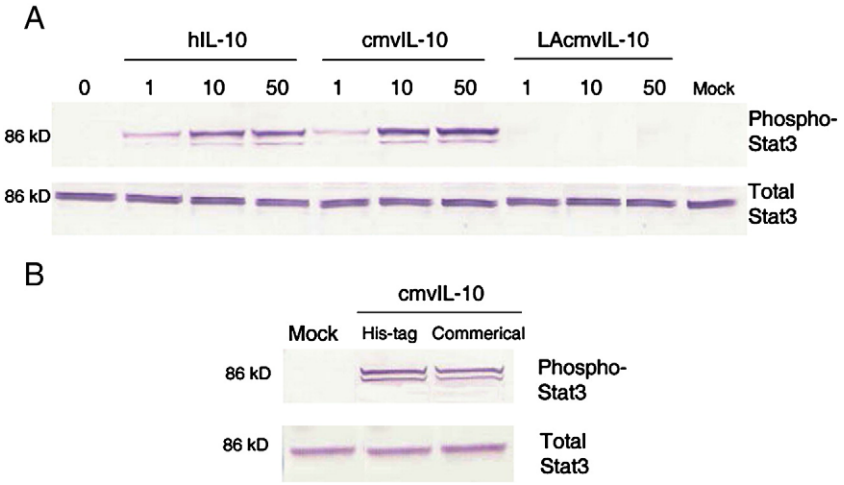

Fig. 2. cmvIL-10 activates Stat3 via binding to the cellular IL-10 receptor (A). Daudi B cells were treated with the indicated doses (ng/ml) of hIL-10, cmvIL-10 or LAcmvIL-10, then cell lysates were analyzed by Western blotting with antibodies to total Stat3 or phosphorylated Stat3 (Y705). Total Stat3 antibodies detect unmodified protein and serve as a loading control to ensure all lanes contained the same amount of protein. Antibodies were from Cell Signaling Technology and detection was via the Western Breeze system (Invitrogen). The well labeled Mock was treated with the volume of mock protein preparation equivalent to the highest dose of LAcmvIL-10. This result is representative of three separate experiments. (B) Stat3 phosphorylation in Daudi B cells treated with either 10 ng/ml of his-tagged cmvIL-10 or commercial cmvIL-10 from R&D Systems.

available purified cmvIL-10 protein to ensure consistency. Because the LAcmvIL-10 protein produced here still contains the his-tag, we wished to confirm that the epitope tag did not interfere with the stimulatory properties of the viral cytokine. As shown in Fig. 2B, Stat3 activation was observed in Daudi B cells treated with either purified recombinant his-tagged cmvIL-10 or with commercial cmvIL-10 protein (without a his-tag) purchased from R&D Systems. The results demonstrate that the his-tagged cytokine has the same biological functions as the commercially available purified cmvIL-10 and rule out the possibility that LAcmvIL-10 fails to activate Stat3 due to interference from the his-tag. Taken together, these experiments indicate that while cmvIL-10 shares functions of hIL-10, including stimulation of B cells, the latency-associated form of the viral cytokine differs significantly.

Given that hIL-10 is a growth factor for B lymphocytes, we asked whether cmvIL-10 treatment could stimulate production of hIL-10 from Daudi B cells. Cells were treated with varying doses of cmvIL-10, LAcmvIL-10, or mock protein preparation for 48 h, and then the supernatants were harvested and analyzed for the presence of hIL-10 by ELISA. As shown in Fig. 3A, hIL-10 production by Daudi B cells was increased in a dose-dependent manner by cmvIL-10 treatment. Basal levels of hIL-10 production were increased by nearly sevenfold in the presence of 50 ng/ml cmvIL-10. No detection of cmvIL-10 was observed using the hIL-10 ELISA reagents, eliminating the possibility that the increase was due to antibody cross reactivity with the viral cytokine added to the culture medium. Once again, no stimulatory activity was detected for LAcmvIL-10, which did not trigger autocrine production of hIL-10 at any dose.

Because we failed to observe stimulatory activity by LAcmvIL-10, we wished to demonstrate that the protein was in fact biologically active. Since both hIL-10 and cmvIL-10 are potent inhibitors of monocyte cytokine synthesis, we tested the ability of LAcmvIL-10 to inhibit TNFα production by human myeloid

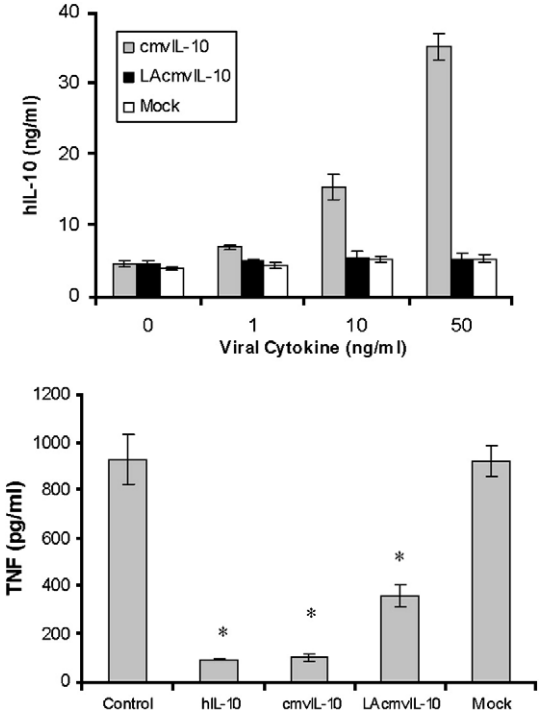

Fig. 3. cmvIL-10 induces endogenous cellular hIL-10 production. (A) Daudi B cells were treated with the indicated concentrations of cmvIL-10, LAcmvIL-10, or mock protein for 48 h, and then supernatants were assayed by sandwich ELISA (R&D Systems). Shown are mean concentrations from triplicate cultures and results are representative of two separate experiments. (B) Inhibition of TNFα by LAcmvIL-10. THP-1 myeloid cells were stimulated with LPS in the presence or absence of hIL-10, cmvIL-10 or LAcmvIL-10. After 24 h, supernatants were collected and analyzed by sandwich ELISA. Shown are mean concentrations from triplicate cultures and results are representative of three separate experiments. Error bars indicate standard deviation from the mean value for three replicate data points. Statistical analysis showed that TNFα levels decreased significantly in the presence of hIL-10, cmvIL-10 or LAcmvIL-10 (*$P<.005$).

cells. THP-1 myeloid cells were treated with the indicated cytokines prior to stimulation with 10 ng/ml LPS for 24 h. As shown in Fig. 3B, control cells produced a significant amount of TNFα when stimulated with LPS. In comparison, the amount of TNFα produced was decreased by about 90% when the cells were pre-incubated with either hIL-10 or cmvIL-10 and about 60% with LAcmvIL-10. These data demonstrate that together with hIL-10 and cmvIL-10, LAcmvIL-10 is a biologically active protein with immune suppressive function on myeloid lineage cells.

Finally, since cmvIL-10 induced production of cellular hIL-10 from B cells, we asked whether there was any impact on the level of expression of the IL-10 receptor (IL-10R). Daudi B cells were incubated with cmvIL-10 or LAcmvIL-10 for 48 h, then stained with antibodies to the cellular IL-10 receptor and examined via flow cytometry. Cells treated with cmvIL-10 exhibited a modest increase in IL-10R expression (Fig. 4). The mean fluorescence intensity increased by nearly 40% over control mock-treated cells, suggesting that more receptors were present on the cell surface after incubation with the viral cytokine. Treatment with LAcmvIL-10 did not significantly impact IL-10R surface levels. For comparison, the impact of the viral cytokines on two other cell surface molecules was also examined. CD19 is a B cell marker, and the amount of this protein in the cell surface was the same regardless of cell treatment. HLA-DR is a haplotype of the human MHC class II complex, and this protein was also found to be highly expressed on Daudi B cells. Despite reports that cmvIL-10 and LAcmvIL-10 can reduce MHC expression on monocytes and dendritic cells (Chang et al., 2004; Raftery et al., 2004; Spencer et al., 2002), no impact on MHC class II levels was observed here. These results demonstrate that cmvIL-10 causes up-regulation of the cellular hIL-10R without affecting CD19 or MHC class II expression levels.

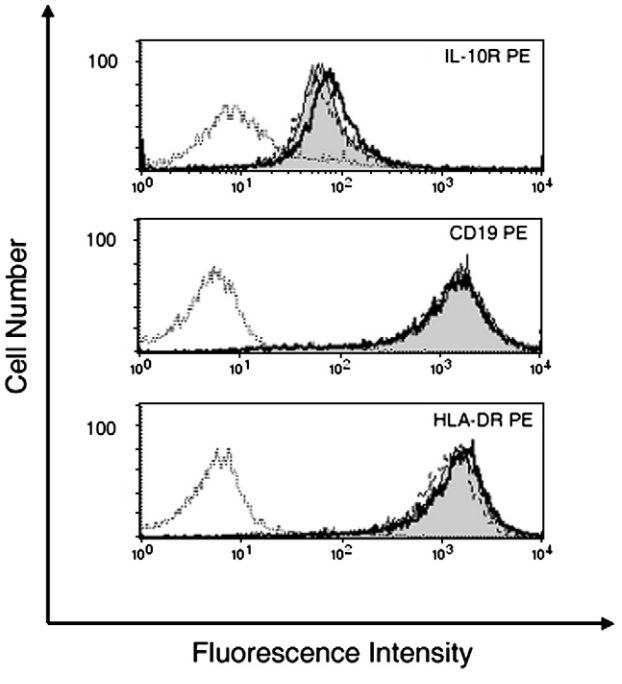

Fig. 4. Up-regulation of cellular IL-10R by cmvIL-10. A) Daudi B cells were treated with 50 ng/ml cmvIL-10 (solid line), LAcmvIL-10 (dashed line), or Mock preparation (control, gray shaded histogram) for 48 h before staining with the indicated phycoerythrin-conjugated antibodies and analysis by flow cytometry. The dotted line indicates mock-treated cells stained with the appropriate isotype control antibody, and relative fluorescence intensity is shown on the *X*-axis in log scale. These results are representative of three separate experiments.

Discussion

Utilization of IL-10 is a common mechanism for intracellular pathogens to suppress or delay the immune response and establish productive infection in the host (Redpath et al., 2001). cmvIL-10 has previously been demonstrated to inhibit PBMC proliferation, suppress inflammatory cytokine production, and down-regulate MHC expression (Spencer et al., 2002). Recent studies also show that cmvIL-10 inhibits dendritic cell maturation and migration (Chang et al., 2004; Raftery et al., 2004), effects that are likely to significantly hamper the cell-mediated immune response to HCMV infection. Our study demonstrates that cmvIL-10 has the ability to stimulate B cell proliferation and induce enhanced production of endogenous hIL-10. This represents the first report of stimulatory activity toward B cells associated with the HCMV encoded IL-10 homolog. This suggests that not only is the immune response to HCMV infection suppressed when cmvIL-10 is present, but the viral cytokine may actually initiate a Th_2 type of response in the host. Since humoral immunity is less effective at controlling virus infection than the cell-mediated response, stimulation of B cells by cmvIL-10 may delay the appropriate anti-viral immune response, allowing HCMV time to establish persistent infection. Although the study would have been more physiologically relevant had it been conducted with primary B cells, attempts to isolate primary B cells from human blood samples were hindered by low yields. As a result, we focused our efforts on the effect of the viral cytokines on B cell lines. While the data shown here are for the Daudi B cell line, we also observed that cmvIL-10 could stimulate proliferation and Stat3 activation for three other B cell lines: Jijoye (EBV-positive B cell lymphoma), Ramos (EBV-negative B cell lymphoma) and JMI (EBV negative pre-B cell lymphoma) cell lines (data not shown). While we cannot confirm that cmvIL-10 would have these same stimulatory effects on primary B cells, the observation that several different B cell lines were similarly affected supports the notion that the viral cytokine is likely to stimulate B cells *in vivo* in the context of viral infection.

Both inhibitory and stimulatory effects of IL-10 are mediated through the cellular IL-10 receptor. Despite their considerable sequence divergence, the crystal structure revealed that cmvIL-10 and hIL-10 share similar contact points with and binding affinities for the cellular IL-10 receptor (Jones et al., 2002). Engagement of the IL-10 receptor by cmvIL-10 has previously been shown to activate Stat3 in various cell types (Kotenko et al., 2000; Spencer, 2007). In B lymphocytes, we have observed activation of Stat3 that correlates with cmvIL-10 stimulatory activity. It is of note that LAcmvIL-10 did not induce Stat3 activation. This result is not surprising considering that the truncated latency-associated protein lacks a significant number of IL-10R contact residues located at the carboxy-terminus of the full-length cmvIL-10 protein (Jones et al., 2002). This is also consistent with the finding

that the currently observed biological activity of LAcmvIL-10, namely down-regulation of class II MHC expression on myeloid cells, occurs in an IL-10R independent fashion. Further investigation will be required to identify the alternative receptor and determine the mechanism by which LAcmvIL-10 contributes to class II MHC down-regulation.

Although we observed up-regulation of both hIL-10 production and the expression level of the cellular IL-10 receptor, no change in MHC class II protein levels was observed on B cells in this study. Previous reports indicate that hIL-10 increases MHC class II expression, although this activity was not conserved by the Epstein–Barr virus encoded vIL-10 (Go et al., 1990). Given the many strategies employed by HCMV for interfering with the MHC antigen presentation system (Basta and Bennink, 2003; Hengel et al., 1998; Mocarski, 2002), it is perhaps to be expected that the viral cytokine would fail to increase MHC class II expression.

Human IL-10 plays a pivotal role in regulation of the immune response and greatly impacts the outcome of virus infection. Recent evidence suggests that the presence of IL-10 is one of the single most important factors contributing to ineffective antiviral responses, and early neutralization of IL-10 activity can prevent persistent virus infection (Brooks et al., 2006). The presence of a viral IL-10 homolog encoded in the genome of HCMV and other viruses is clearly a significant benefit for the establishment of latency and/or persistent infection. Our results demonstrate that not only does cmvIL-10 dampen the inflammatory immune response but it can also stimulate the humoral response, leading to even greater interference with virus clearance. The existence of a second, latency-associated form of cmvIL-10 that lacks these immunomodulatory properties suggests that these viral cytokines may play a role in the establishment of or reactivation from latency. HCMV has capitalized on the immunoregulatory properties associated with hIL-10 in three ways: enabling the full spectrum of IL-10 biological activities by triggering production of the human cytokine, invoking a smaller subset of these properties through production of cmvIL-10 during productive infection, and finally, use of a more limited range of immunosuppressive activities through production of LAcmvIL-10 during latent infection. Future studies will be required to investigate the full range of biological activities of LAcmvIL-10 and to understand the relationship between cmvIL-10 and LAcmvIL-10 during the infection-latency cycle.

Materials and methods

Cells and cytokines

Daudi human B cell lymphoblastoma cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and maintained in RPMI 1640 tissue culture medium (Mediatech, Inc., Herndon, VA) supplemented with 4.5 g/L glucose, 1.5 g/L sodium bicarbonate, 2 mM L-glutamine, 1 mM sodium pyruvate, 10 mM HEPES and 10% fetal bovine serum (FBS; Atlanta Biologicals, Atlanta, GA) in a 37 °C incubator with a humidified 5% CO_2 environment. THP-1 human monocytic cells (ATCC) were likewise maintained in RPMI supplemented as described above. Purified recombinant human IL-4, IL-10 and cmvIL-10 were purchased from R&D Systems (Minneapolis, MN). LAcmvIL-10 and cmvIL-10 were purified by ion-exchange chromatography from lysates of bacterial cells expressing plasmid encoded histidine-tagged cytokine. The concentration of the purified protein was determined via spectrophotometry. Preparations from mock transformed bacterial cell lysates were used as a control.

Cell proliferation

Cells were plated at 5×10^4 per well in white 96-well plates in the presence or absence of cytokine as indicated. Cell growth was measured using the Cell Titer Glo Kit (Promega, Madison, WI). Briefly, a luciferin substrate added to each well was converted to oxyluciferin in the presence of O_2 and ATP. The light produced was measured using a Veritas Microplate Luminometer (Turner Biosystems, Sunnyvale, CA) and was proportional to the amount of ATP present, reflecting the number of viable cells in the well.

Western blotting

Cells were treated with cytokine as indicated, then harvested and incubated with cell lysis buffer (150 mM NaCl, 20 mM HEPES, 1% Triton X-100, 1 mM Na_3VO_4, 1 mM EDTA, and 1 mM PMSF) at 4 °C for 30 min. Cell lysates were clarified by centrifugation, and the supernatants were then analyzed by SDS–PAGE and subsequent Western blotting with polyclonal antiserum directed against either total Stat3 or phospho-Stat3-(Y705) (Cell Signaling Technologies, Danvers, MA) according to the manufacturer's instructions. Alkaline phosphatase-conjugated goat anti-rabbit secondary antibody (Anaspec, San Jose, CA) and Western Blue Substrate (Promega) were utilized for detection.

ELISA

Following cell treatment, supernatants were collected and human cellular IL-10 levels analyzed via sandwich ELISA (R&D Systems). The assay was carried out according to the manufacturer's instructions; resulting samples were analyzed at 450 nm, and cytokine levels determined by linear regression analysis using a standard curve. TNFα levels were similarly measured via sandwich ELISA (R&D Systems) following LPS (lipopolysaccharide; Sigma-Aldrich, St. Louis, MO) and cytokine treatment of THP-1 cells. Statistical analysis was performed with a two-tailed, paired Student's *t* test.

Flow cytometry

Cells were treated for 48 h, then washed and suspended in FACS buffer (PBS + 0.5% BSA + 1 mM EDTA) before staining with phycoerythrin conjugated antibodies specific for CD19, IL-10 receptor, HLA-DR (Class II MHC) or the appropriate isotype control (IgG_{2a} for CD19 and HLA-DR, IgG_1 for IL-10R; BD Pharmingen, San Diego, CA). After staining for 1 h on ice, cells were washed twice and then resuspended in 300 μl FACS buffer. Data was collected immediately with the BD FACSCalibur and analyzed using CellQuest Pro software.

Acknowledgments

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Laboratory electives within the molecular biology emphasis and molecular methods

	Biology 324-325 Molecular Ecology	Biology 333-334 Endocrinology	Biology 346-347 or 341-342 General or Medical Microbiology	Biology 355-356 Developmental Biology	Biology 443-444 Immunology	Biology 458 Techniques in Light & Electron Microscopy	Biology 485-486 Molecular Genetics & Biotechnology
Aseptic Technique			√ G, M		√		√
Sterile Technique						√	√
Mammalian Tissue Culture						√	√
DNA Transfection / Transgenesis				√		√	√
Ectopic Gene Expression				√			
Enhancer Traps				√			
Transplantation / Microinjection				√			
siRNA Transfection				√		√	
Bacterial Culture / Plating Methods			√ G, M		√		√
Plasmid Prep							√
Genomic DNA Prep	√						√
Restriction Digestion	√		√G				√
DNA Ligation	√						√
Agarose Gel Electrophoresis	√		√G		√		√
RNA prep		√					√
Transposon mutagenesis							√
Apoptosis Assay				√			
Ion-Exchange Chromatography					√		√
SDS-PAGE		√			√		√
Western Blot		√			√		√
ELISA		√	√M		√		
PCR, PCR cleanup	√	√	√G				√
qPCR		√					√
Immunochemistry		√			√		
in situ Hybridization				√			
in vitro Transcription				√			
Immuno-fluorescence				√	√	√	
Flow Cytometry					√	√	√
Cell Lineage Tracing				√			
Chemical Genetics / Cell Signaling				√			
DNA sequencing							√
Southern Blotting and Hybridization							√

UNIVERSITY OF SAN FRANCISCO
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Biology – The Science of Life
Molecular Biology Emphasis

This area of emphasis within the Biology major is designed to prepare students for positions in academic, clinical or industrial biotechnology laboratories. It also prepares students for graduate study in genetics, molecular biology and other fields of the life sciences.

The Molecular Biology emphasis includes most of the same requirements as the standard Biology major. The difference is that a molecular course is substituted for the field/ecology course requirement, and specific upper division elective courses must be taken.

Molecular Biology Emphasis Requirements (66-73 units):

- General Biology I and II
- Cell Physiology
- Genetics
- Evolution
- General or Medical Microbiology
- Molecular Genetics and Biotechnology
- Fundamentals of Biochemistry (or Biochemistry I and II)
- Biology Seminar (1 unit)
- 8 units of upper division electives from the following (must include at least one laboratory course): Endocrinology, Virology, Development, Biology of Cancer (SL), Molecular Medicine, Molecular Biology, Immunology, Techniques in Cell Biology, Drug Discovery in Biotechnology, Research for Advanced Undergraduates, Biology Honor's Thesis. +

Supporting Courses
- General Chemistry I and II
- Organic Chemistry (1 or 2 semester option)
- Organic Chemistry Lab I
- Biostatistics
- Physics I and II

Sample Curriculum (depends on Core and language placement)

	Fall	***Spring***
Freshman Year	General Biology I General Chemistry I Core Writing Requirement Core Public Speaking	General Biology II General Chemistry II Core: Biostatistics Core Writing Requirement
Sophomore Year	Cell Physiology Organic Chemistry I* + lab Biology Seminar (1 unit) Core Core or Language Requirement	Genetics Organic Chemistry II Core/General Elective/Minor Core or Language Requirement
Junior Year	Microbiology Physics I Core General Elective/Minor	Fundamentals of Biochemistry Physics II Core General Elective/Minor
Senior Year	Evolution Upper-Division Biology Elective Core General Elective/Minor	Molecular Genetics and Biotechnology Upper-Division Biology Elective Core/General Elective/Minor General Elective/Minor

Students must declare the Molecular Biology Emphasis by the end of the junior year.

*curriculum will be slightly modified if one-semester Organic Chemistry option is chosen (see your advisor)

26 August 2013

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Biology – The Science of Life
Molecular Biology Emphasis

This area of emphasis within the Biology major is designed to prepare students for positions in academic, clinical or industrial biotechnology laboratories. It also prepares students for graduate study in genetics, molecular biology and a range of fields of the life sciences.

The Molecular Biology emphasis includes most of the same requirements as the standard Biology major. The difference is that a molecular course is substituted for the field/ecology course requirement, and the upper-division science requirements include one additional course: Biochemistry.

Molecular Biology Emphasis Requirements (66-73 units):

- General Biology I and II
- Cell Physiology
- Genetics
- Evolution
- Molecular Biology
- Fundamentals of Biochemistry (or Biochemistry I and II)
- Biology Seminar (1 unit)
- 16 units of upper division electives from the following (must include at least three laboratory courses): Molecular Ecology, Endocrinology, Virology, General OR Medical Microbiology, Developmental Biology, Biology of Cancer (SL), Molecular Medicine, Immunology, Techniques in Light and Electron Microscopy, Molecular Genetics & Biotechnology, Drug Discovery in Biotechnology, Research for Advanced Undergraduates, Biology Honor's Thesis.

Supporting Courses
- General Chemistry I and II
- Organic Chemistry (1 or 2 semester option)
- Organic Chemistry Lab I
- Biostatistics
- Physics I and II

Sample Curriculum (depends on Core and language placement)

	Fall	***Spring***
Freshman Year	General Biology I General Chemistry I Core Writing Requirement Core Public Speaking	General Biology II General Chemistry II Core: Biostatistics Core Writing Requirement
Sophomore Year	Cell Physiology Organic Chemistry I* + lab Biology Seminar (1 unit) Core Core or Language Requirement	Genetics Organic Chemistry II Core/General Elective/Minor Core or Language Requirement
Junior Year	Molecular Biology Physics I Core General Elective/Minor	Upper-Division Biology Elective Physics II Core General Elective/Minor
Senior Year	Evolution Fundamentals of Biochemistry Upper-Division Biology Elective Core	Upper-Division Biology Elective Upper-Division Biology Elective Core/General Elective/Minor General Elective/Minor

Students must declare the Molecular Biology Emphasis by the end of the junior year.

*curriculum will be slightly modified if one-semester Organic Chemistry option is chosen (see your advisor)

17 September 2015

Initial Report

Last Modified: 05/06/2016

1. I am able to describe structure-function relationships at the following levels:

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	molecular	0	1	15	14	30	3.43
2	cellular	0	0	17	12	29	3.41
3	organismal	0	1	15	14	30	3.43
4	ecological	1	3	16	10	30	3.17

Statistic	molecular	cellular	organismal	ecological
Min Value	2	3	2	1
Max Value	4	4	4	4
Mean	3.43	3.41	3.43	3.17
Variance	0.32	0.25	0.32	0.56
Standard Deviation	0.57	0.50	0.57	0.75
Total Responses	30	29	30	30

2. I am able to

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	integrate molecular, cellular, organismal and ecological principles to understand and describe biological systems.	0	0	18	12	30	3.40

Statistic	integrate molecular, cellular, organismal and ecological principles to understand and describe biological systems.
Min Value	3
Max Value	4
Mean	3.40
Variance	0.25
Standard Deviation	0.50
Total Responses	30

3. I am able to

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	explain the chromosomal and molecular basis of heredity	0	0	15	15	30	3.50
2	explain the genetic basis of evolution	0	1	12	17	30	3.53
3	apply evolutionary principles to understanding biological systems	0	1	14	15	30	3.47

Statistic	explain the chromosomal and molecular basis of heredity	explain the genetic basis of evolution	apply evolutionary principles to understanding biological systems
Min Value	3	2	2
Max Value	4	4	4
Mean	3.50	3.53	3.47
Variance	0.26	0.33	0.33
Standard Deviation	0.51	0.57	0.57
Total Responses	30	30	30

4. I am able to

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	understand primary research articles and other scientific publications in biology	0	0	10	20	30	3.67
2	critically evaluate primary research articles and other scientific publications in biology	0	1	12	17	30	3.53

Statistic	understand primary research articles and other scientific publications in biology	critically evaluate primary research articles and other scientific publications in biology
Min Value	3	2
Max Value	4	4
Mean	3.67	3.53
Variance	0.23	0.33
Standard Deviation	0.48	0.57
Total Responses	30	30

5. I am familiar with

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	laboratory techniques used in biology	0	1	13	16	30	3.50
2	field methods used in biology (answer only if you took one or more field biology courses)	1	3	15	8	27	3.11
3	how to apply the scientific process to test hypotheses through experimentation	0	1	13	16	30	3.50

Statistic	laboratory techniques used in biology	field methods used in biology (answer only if you took one or more field biology courses)	how to apply the scientific process to test hypotheses through experimentation
Min Value	2	1	2
Max Value	4	4	4
Mean	3.50	3.11	3.50
Variance	0.33	0.56	0.33
Standard Deviation	0.57	0.75	0.57
Total Responses	30	27	30

6. I am aware of

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	a variety of careers and professions in the biological sciences	2	4	13	11	30	3.10

Statistic	a variety of careers and professions in the biological sciences
Min Value	1
Max Value	4
Mean	3.10
Variance	0.78
Standard Deviation	0.88
Total Responses	30

7. The following courses prepared me for biology courses that followed them.

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	General Biology	1	1	10	18	30	3.50
2	Cell Physiology	1	7	12	10	30	3.03
3	Genetics	2	4	14	10	30	3.07

Statistic	General Biology	Cell Physiology	Genetics
Min Value	1	1	1
Max Value	4	4	4
Mean	3.50	3.03	3.07
Variance	0.53	0.72	0.75
Standard Deviation	0.73	0.85	0.87
Total Responses	30	30	30

8. The following courses provided a foundation for my understanding of biology.

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	General Chemistry	3	6	12	9	30	2.90
2	Organic Chemistry	0	3	9	18	30	3.50

Statistic	General Chemistry	Organic Chemistry
Min Value	1	2
Max Value	4	4
Mean	2.90	3.50
Variance	0.92	0.47
Standard Deviation	0.96	0.68
Total Responses	30	30

9. The upper-division courses I took

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	enhanced my breadth of understanding in biology	0	0	9	21	30	3.70
2	were relevant to my career goals	1	2	11	16	30	3.40
3	were courses that I wanted to take	1	3	7	19	30	3.47
4	were chosen from a significant number of options	2	7	14	7	30	2.87

Statistic	enhanced my breadth of understanding in biology	were relevant to my career goals	were courses that I wanted to take	were chosen from a significant number of options
Min Value	3	1	1	1
Max Value	4	4	4	4
Mean	3.70	3.40	3.47	2.87
Variance	0.22	0.59	0.67	0.74
Standard Deviation	0.47	0.77	0.82	0.86
Total Responses	30	30	30	30

10. My breadth and depth of understanding in biology was enhanced by

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	the laboratories associated with upper-division courses	0	1	14	15	30	3.47
2	the posters, projects and presentations associated with upper-division courses	0	4	19	7	30	3.10
3	the upper-division field biology course/s I took (answer only if you took at least one field course).	3	1	12	13	29	3.21

Statistic	the laboratories associated with upper-division courses	the posters, projects and presentations associated with upper-division courses	the upper-division field biology course/s I took (answer only if you took at least one field course).
Min Value	2	2	1
Max Value	4	4	4
Mean	3.47	3.10	3.21
Variance	0.33	0.37	0.88
Standard Deviation	0.57	0.61	0.94
Total Responses	30	30	29

11. The biology courses I took

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	required an appropriate amount of work and effort	2	1	14	13	30	3.27
2	addressed underlying evolutionary themes	0	2	15	13	30	3.37
3	incorporated USF's Mission and values	2	3	15	10	30	3.10

Statistic	required an appropriate amount of work and effort	addressed underlying evolutionary themes	incorporated USF's Mission and values
Min Value	1	2	1
Max Value	4	4	4
Mean	3.27	3.37	3.10
Variance	0.69	0.38	0.71
Standard Deviation	0.83	0.61	0.84
Total Responses	30	30	30

12. My grade point average in biology

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	is the expected result of my ability and effort	0	8	10	12	30	3.13

Statistic	is the expected result of my ability and effort
Min Value	2
Max Value	4
Mean	3.13
Variance	0.67
Standard Deviation	0.82
Total Responses	30

13. As a biology major at USF, I

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	participated in biological and/or science club/s	1	12	10	7	30	2.77
2	made friends who shared my goals	3	0	14	13	30	3.23

Statistic	participated in biological and/or science club/s	made friends who shared my goals
Min Value	1	1
Max Value	4	4
Mean	2.77	3.23
Variance	0.74	0.81
Standard Deviation	0.86	0.90
Total Responses	30	30

14. During my undergraduate years, I participated in research

#	Answer		Response	%
1	Yes		16	53%
2	No		14	47%
	Total		30	100%

Statistic	Value
Min Value	1
Max Value	2
Mean	1.47
Variance	0.26
Standard Deviation	0.51
Total Responses	30

15. I participated in research in the Biology Department

#	Answer		Response	%
1	Yes		5	31%
2	No		11	69%
	Total		16	100%

Statistic	Value
Min Value	1
Max Value	2
Mean	1.69
Variance	0.23
Standard Deviation	0.48
Total Responses	16

16. I participated in research in another department at USF

#	Answer		Response	%
1	Yes		4	25%
2	No		12	75%
	Total		16	100%

Statistic	Value
Min Value	1
Max Value	2
Mean	1.75
Variance	0.20
Standard Deviation	0.45
Total Responses	16

17. I participated in research at UCSF or at another local institution

#	Answer		Response	%
1	Yes		6	38%
2	No		10	63%
	Total		16	100%

Statistic	Value
Min Value	1
Max Value	2
Mean	1.63
Variance	0.25
Standard Deviation	0.50
Total Responses	16

18. I participated in research through a structured program such as a summer internship

#	Answer		Response	%
1	Yes		7	44%
2	No		9	56%
	Total		16	100%

Statistic	Value
Min Value	1
Max Value	2
Mean	1.56
Variance	0.26
Standard Deviation	0.51
Total Responses	16

19. Having participated in a research project,

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	I understand how research is accomplished	0	1	6	9	16	3.50
2	I have enriched my understanding of biology	0	1	5	9	15	3.53

Statistic	I understand how research is accomplished	I have enriched my understanding of biology
Min Value	2	2
Max Value	4	4
Mean	3.50	3.53
Variance	0.40	0.41
Standard Deviation	0.63	0.64
Total Responses	16	15

20. I have been to the Fletcher Jones Microscopy Center (Harney 103) at least once

#	Answer		Response	%
1	Yes		14	47%
2	No		16	53%
	Total		30	100%

Statistic	Value
Min Value	1
Max Value	2
Mean	1.53
Variance	0.26
Standard Deviation	0.51
Total Responses	30

21. Regarding my time in the Fletcher Jones Microscopy Center (select all that apply);

#	Question	I was given a tour of the Center	I was shown a demonstration of equipment in the Center	I used equipment in the Center as part of a class	I used equipment in the Center as part of a research project	Total Responses
1	Please select all that apply	7	11	10	1	29

Statistic	Please select all that apply
Min Value	1
Max Value	4
Total Responses	14

22. My academic adviser

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	was available, informed and helpful	2	1	13	13	29	3.28

Statistic	was available, informed and helpful
Min Value	1
Max Value	4
Mean	3.28
Variance	0.71
Standard Deviation	0.84
Total Responses	29

23. The professors in the Department of Biology

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	are excellent teachers	1	0	6	22	29	3.69
2	have a positive attitude toward students	1	0	10	18	29	3.55

Statistic	are excellent teachers	have a positive attitude toward students
Min Value	1	1
Max Value	4	4
Mean	3.69	3.55
Variance	0.44	0.47
Standard Deviation	0.66	0.69
Total Responses	29	29

24. My degree in biology

#	Question	Strongly Disagree	Disagree	Agree	Strongly Agree	Total Responses	Mean
1	has prepared me for the next step in my life	1	2	11	15	29	3.38

Statistic	has prepared me for the next step in my life
Min Value	1
Max Value	4
Mean	3.38
Variance	0.60
Standard Deviation	0.78
Total Responses	29

25. In the coming year,

#	Question	attend graduate school	attend professional school (e.g., medical, dental, pharmacy, optometry)	work in a biology-related field	work in a field that is not related to biology	other	Total Responses
1	I plan to	6	20	11	3	4	44

Statistic	I plan to
Min Value	1
Max Value	5
Total Responses	29

26. Looking beyond the coming year,

#	Question	researcher in the biological sciences	health care professional	teacher	Lab technician	other	Total Responses
1	I plan to become a	3	24	1	1	3	32

Statistic	I plan to become a
Min Value	1
Max Value	5
Total Responses	29

27. We welcome your comments, including those related to upper-division course offerings, intersession and summer biology courses at USF, advising support, career development support, etc. **PLEASE do not refer to specific faculty members**

Text Response
It would be really helpful for students to have the same advisor for all for years if possible. Also, I would appreciate more activities within the major to build friendships!
I believe the upper division classes were tremendously helpful in preparing me for dental school. The faculty as well as the lab components really gave me the confidence and knowledge that I will need later in my career. In addition, the support I've received from faculty here is amazing. All my teachers in the biology department were very accessible during the week. I would suggest having a wider selection of biology classes or even more sections since a lot of Juniors and some Seniors had trouble getting their designated classes for graduation school. Overall, I am very satisfied with my education here at USF.
I think that higher priority should be given to graduating seniors for registration because I had very limited choices when I was signing up for my last two semesters at USF because many spots were filled up before I was approved for registration. It is unfair that many juniors had earlier registration than many graduating seniors. Also, some sort of career development support should be mandatory every year/semester so that you can help students continue on the right track in terms of career planning and preparedness.
I never had a teacher that I did not like or that did not motivate me to learn the information and apply it to practical terms. My professors have always been incredibly helpful and encouraging about learning the information. I did have one professor who did not fully prepare you before the exams and the exams were not a reflection of what exactly what was learned in class, which made it extremely hard to study for the exams and therefore, our grades suffered tremendously no matter how hard and long you studied. However, overall I am incredibly satisfied with my academic experience at USF and I am so grateful for the work that the professors put towards their jobs to help us learn the material that broadens our perspective of the biological world.
I wish there were more courses offered during intersession and during the fall semester. I also wished i knew that I had the option of switching my bio advisor at an early point
I thought having Evolution as a capstone course was a fantastic way to wrap up the years of underlying biology. Overall, I was extremely satisfied with my academic career here at USF.
It would be helpful to have a statistics class taught within the biology department itself using examples from primary literature.
It would be great if we had a diverse staff that knew how to interact with students of color.
I had a bad experience doing research in a USF lab (not biology) and it subsequently led me to not pursue science as a career in the immediate future
I LOVED THIS PROGRAM AND THIS DEPARTMENT!!! I will miss you all!

Statistic	Value
Total Responses	10