Overall Project Title: Cytokine Production and Urinary Excretion in Free-Ranging Orangutan

Subproject Title: Ex vivo Orangutan Cytokine Production: Species-Specificity and Endotoxin-Sensitivity

Lyle L. Moldawer, Ph.D.
Laboratory of Inflammation Biology
Department of Surgery
University of Florida College of Medicine

Erin Vogel, Ph.D.
Center for Human Evolutionary Studies
Department of Anthropology
Rutgers University

Rationale and Significance

It is well recognized that humans are exquisitely sensitive to the inflammatory properties of bacterial lipopolysaccharide (LPS). LPS or endotoxin is the compound in bacteria by which we recognize the presence of an infection. Doses of bacterial endotoxin or LPS as low as 4 ng/kg BW in humans can produce a systemic inflammatory response including fever, hypotension, tachycardia and sickness syndromes. Plasma concentrations of TNFa and IL-1b peak at 90 minutes and more distal cytokines, such as IL-6, IL-8, IL-10 and cytokine antagonists, such as sTNFRI and sTNFRII, peak at 4-8 hours after exposure to LPS. Not surprisingly, endotoxin responsiveness varies dramatically among different mammals. C57BL/6 mice for example are at least four to five logs less sensitive to in vivo LPS than humans, with doses of 10-1000 ug/kg BW required to produce an equivalent in vivo response. The response by nonhuman primates (NHP) to endotoxin has not been systematically studied. In the great apes, endotoxin responsiveness is only known for the chimpanzee (*Pan sp.*). Studies by van der Poll and colleagues have shown that chimpanzee share with humans, a very high sensitivity to LPS with doses equivalent to humans (4 ng/kg BW). Comparable studies have not been performed in orangutan (*Pongo sp.*).

Studies conducted in other nonhuman primates, however, have demonstrated considerable variation in their responsiveness to endotoxin. In studies we conducted several years ago with baboons (*Papio sp.*), endotoxin doses as high as 500 mg/kg were required to produce significant hypotension and an inflammatory response. In contrast, rhesus monkeys (*Macaca mulatta)* are more sensitive to endotoxin, although their responsiveness does not approach that seen in either chimpanzee or man. Doses ranges from 1-5 ug/kg BW are required to produce a systemic inflammatory response. The mechanisms behind the five-six log differences in the sensitivity to bacterial endotoxin among primate species remains unknown. At present, there are two competing hypotheses. The first focuses on the direct interaction between LPS and the CD14/TLR4/MD1 complex, and the relative affinities for bacterial LPS. In 2008, the TLR4 and CD14 receptors were sequenced for all of the great apes as well as for several old world and new world monkeys, and phylogenic trees created. Heterogeneity in the extracellular domain of the TLR4 receptor could explain many of the different affinities for *Macaca* and *Papio*, although functional assays have not been performed. Similarly, LBP and MD2 have not been sequenced for these species, so a complete picture is still lacking. An alternative explanation is that species with resistance to endotoxin responsiveness have blood factors that bind endotoxin and interfere with its interactions with the CD14/TLR4/MD2 complex. BPI was first described in 1989 as one such protein. Another such molecule, hemopexin, has been postulated to play this role and to explain the relative different sensitivities to endotoxicosis in different species.

Hypothesis: A high degree of endotoxin-responsiveness and cytokine production is shared by orangutan and man.

Specific Aims: (1) to determine cross-reactivity among orangutan cytokines and human immunoassay reagents, (2) to determine ex vivo cytokine production to bacterial endotoxin by whole blood obtained from orangutan. (3) to measure hemopexin and other acute phase reactant proteins that bind endotoxin (LBP, BPI) in the plasma of orangutan.

We intend to collect fresh waste blood from healthy orangutans that are being bled as part of a general health physical, and will stimulate the blood ex vivo with varying amounts of endotoxin. We will measure cytokines in the blood following LPS stimulation. The amount of LPS used to stimulate the whole blood will vary from 1 ngs/ml to 10 μg/ml.

Research Study Description

The proposed experiments require a single blood collection, performed at the time of a semi-annual physical examination. We will collect a single blood sample from an orangutan and if possible, would like to obtain three replicates from different animals. The animal(s) in question needs to be in good health with no known active inflammatory process, no history of primary or acquired immune deficiency, and aged in the equivalent of a sexually mature, but not 'aged' human adult. The investigators request that no additional procedures be performed on the animals to collect the single blood sample, other than to collect blood that would normally be collected for the requested studies, as part of the standard care of the animal, or in the opinion of the veterinarians, an additional collected sample poses no risk to the animal.

These preliminary studies are seeking at the minimum one blood sample from an orangutan, collected at the time the animal undergoes a physical examination for general health. Any animals with any inflammatory process are not considered. If possible, a total of three independent samples are desirable.

A single blood collection of 4 mls of whole blood is obtained by venepuncture. The blood can be collected from either a central venous/arterial access or by peripheral venous access (such as the antecubita fossa) being used to flush the line. Or if the individual site permits it, an extra 4 mls collected. The blood can either be collected directly into a syringe and immediately transferred to a 4 ml heparinized green top tube (Becton-Dickinson cat#367871, 367872) or directly into the heparinized green top tube. The blood is immediately mixed by inverting the tube several times and immediately placing on wet ice. The sample should be placed on ice and delivered to the Laboratory at the University of Florida, Shands Hospital by overnight courier.

Prior to collecting the sample, the investigators will receive a Styrofoam box with synthetic ice wet-packs. The wet-packs are kept in the refrigerator until use. The green top tube is placed in zip-lock bag with the enclosed insulation, and then placed in the Styrofoam box with the wet-packs. Additional insulation is added to the top, the box is sealed and the enclosed fedex form is attached to the box and shipped priority overnight. (The cost of shipping the original box and its return shipment of the sample is paid for by the Laboratory). Detailed instructions including

shipping materials, wet-packs and return fedex labels will be provided to the site, once IACUC approval is obtained. It is understood that the shipping institution is aware of the precautions and labeling associated with the shipment of potentially infectious materials. Mr. Abouhamze will contact the appropriate representative of the institution and address any concerns.

Once the four ml sample is received at the laboratory, 100 ul of well mixed blood is pipetted into 24 wells of a 96 flat-bottom microtiter plate, and endotoxin is added to the wells in triplicate (1 ngs/ml – 10,000 ngs/ml). Samples are incubated at 37° C in room air in a wig-wag for 18 hrs. The plate is then centrifuged at 1500 x g for 8 minutes and the plasma removed from each well and frozen at -70° C, until cytokines are measured. The remaining blood is centrifuged at 1500 x g for 8 minutes and the plasma removed and aliquotted and frozen. The remaining erythrocytes are lysed with 10 volumes of EL buffer and the remaining leukocytes are pelleted. One aliquot is frozen in RLT lysis buffer and a second aliquot is fixed for flow cytometry.

The lysed samples are used for determination of nucleic acids, either by sequencing, or by microarray. The fixed samples are used for phenotyping the cell populations by flow cytometry.