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Necator americanus: The *Na*-ASP-2 protein secreted by the infective larvae induces neutrophil recruitment *in vivo* and *in vitro*

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Abstract

The L3-secreted *Ancylostoma* Secreted Protein-2 from the human hookworm *Necator americanus* (*Na*-ASP-2) has been selected as a candidate vaccine antigen in anticipation of clinical trials. Its crystal structure revealed that *Na*-ASP-2 has structural and charge similarities to CC-chemokines, suggesting that it might act as a chemokine mimic when released by the infective larvae during tissue migration. Using the air pouch model of acute inflammation, we found that *Na*-ASP-2 induced a significant leukocyte influx to the skin pouch, mostly comprised of neutrophils (60%) and monocytes (30%) that was transient and resolved in 24 h. Other hookworm larval proteins did not cause any inflammatory leukocytes to migrate into air pouches. *In vitro* chemotaxis assays confirmed our results and demonstrated that leukocyte migration was a direct effect of *Na*-ASP-2 exposure and not caused by other molecules released by host cells in the inflammatory microenvironment or by the expression vector.

Index Descriptors and Abbreviations: *Na*-ASP-2; Neutrophils; Chemotaxis; Vaccine; *Necator americanus*; Air pouch; Nematode

1. Introduction

Human hookworm infection is a leading cause of iron-deficiency anemia and malnutrition with an estimated 600 million cases in the tropical developing world (Bethony et al., 2006). Most hookworms infect a host by penetrating the skin, although some species are orally infective. Third-stage infective larvae (L3) of the canine hookworm, *Ancylostoma caninum,* and the major human hookworm, *Necator americanus*, are developmentally arrested and wait in the soil or on grass. They attach to the host upon direct contact and penetrate the skin via hair follicles, crossing the dermis and eventually entering blood or lymphatic capillaries, where they are carried to the pulmonary microcirculation. Once in the lung, they undergo tracheal migration by penetrating into the alveoli to be swept in mucus up the airways and then down into the gut, where they establish as adult parasites. During tissue invasion, infective L3 of parasitic nematodes encounter physicochemical signals that initiate a programmed chain of developmental events resulting in the successful establishment of a parasitic relationship. When L3 of the canine hookworm *A. caninum* are activated *in vitro*, they release metalloproteases (Hawdon et al., 1995) and other molecules of unknown function referred to as *Ancylostoma* Secreted Protein 1 and 2 (*Ac*-ASP-1, *Ac*-ASP-2; Hawdon et al., 1996, 1999).

Tissue invasion is often associated with granulocyte infiltration. The quantity and quality of inflammatory recruitment following parasitic helminth infection suggest that granulocytes are not simply responding to tissue injury caused by migrating larvae, but they are actively being targeted by molecules secreted by the parasite. Numerous parasite-derived chemotactic factors have been reported to recruit, often selectively, neutrophils or eosinophils (Falcone et al., 2001; Horii et al., 1988; Niwa et al., 1998; Owhashi et al., 1985, 1997; Rubio de Kromer et al., 1998; Tanaka et al., 1979; Tanaka and Torisu, 1978). In the

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few cases in which the mechanism has been studied, it has revealed some degree of interaction between parasite antigens and either ß-integrin receptors or the IL-8 pathway (Falcone et al., 2001; Rubio de Kromer et al., 1998).

Hookworm proteins involved in tissue invasion are particularly good candidate antigens for the development of vaccines and drugs (Hotez et al., 2003). On the basis of *in vitro* data, animal trials and human epidemiological studies (Bethony et al., 2005; Goud et al., 2004; Hotez et al., 2003; Mendez et al., 2005), the L3-secreted *N. americanus* ASP-2 (*Na*-ASP-2) was selected as a vaccine antigen to undergo further development (Goud et al., 2005). As mentioned above, ASP-2 is released by the L3 after invading the host and it is likely to play a role in the transition to parasitism (Hotez et al., 2003). Since the function of ASP-2 is currently unknown, structural studies were initiated to clarify the role of *Na*-ASP-2 as a functional vaccine. Although no protease activity for *Na*-ASP-2 has been detected so far, our finding that anti-*Na*-ASP-2 antibodies inhibit larval migration through skin in an *in vitro* assay (Bethony et al., 2005; Goud et al., 2005) suggests that ASP-2 has an important role in larval host entry and in migration through the tissues before reaching the intestine. Recent crystallography studies have revealed that *Na*-ASP-2 has structural and charge similarities to CC-chemokines (Asojo et al., 2005), suggesting that this molecule might act as a chemokine mimic when released by the infective larvae during tissue migration.

To study the development of chemotaxis, *in vivo* animal models have become important. The study of localized inflammation in tissues is often challenging because it is difficult to isolate the immune response towards a particular insult. Thus, there was value in developing a simple inflammatory model that is restricted to a confined location in which population changes can be more readily monitored and regulatory mechanisms identified. The air pouch model in mice represents an ideal location in which to study the development of an inflammatory reaction. The pouches are generated by the injection of sterile air in the skin of mice. Both the stimulus (inflammatory agent) and the response (cellular and humoral) are generated in a sterile environment and can be easily isolated by lavage. In the present study, we were interested to determine, by use of a murine air pouch system, whether *Na*-ASP-2 had a chemotactic function. We found that *Na*-ASP-2 caused leukocyte recruitment into the inflammatory air pouch of BALB/c mice, and that the infiltrate was mainly comprised of neutrophils (60%) and monocytes (30%). Other hookworm larval secreted proteins, such as *Ac*-MTP-1, did not cause any inflammatory infiltrate. *In vitro* chemotaxis assays using purified neutrophils confirmed that the neutrophil migration was a direct effect of *Na*-ASP-2 exposure and not caused by other chemokines and/or cytokines that may have been released *in vivo* in the inflammatory microenvironment of the air pouch.

2. Materials and methods

2.1. Animals

Female BALB/c were obtained from the National Cancer Institute and used at 6–8 weeks of age. All studies were approved by the Institutional Animal Care and Use Committee at The George Washington University Medical Center.

2.2. Recombinant proteins

The r*Na*-ASP-2 protein was manufactured as previously described (Goud et al., 2005). The recombinant *Ancylostoma caninum* metalloprotease-1 (*Ac*-MTP-1) was used as a control. The recombinant protein was provided by Dr. Bin Zhan of the Department of Microbiology, Immunology, and Tropical Medicine at The George Washington University, and produced as described (Zhan et al., 2002). The rationale for selecting *Ac*-MTP-1 is that this protein is expressed exclusively by the L3 stage of *A. caninum* and is actively secreted into the culture medium *in vitro*, supporting a role in tissue invasion. *Ac*-MTP-1 has significant homology with the *N. americanus* MTP-1 molecule (Daub et al., 2000), which is not available as a recombinant antigen. Both proteins were suspended in sterile phosphate buffer saline (PBS) for use in *in vivo* air pouch inoculations. For *in vitro* chemotaxis assays, proteins were diluted in RPMI supplemented with 1% BSA (Sigma, St. Louis, MO).

2.3. Air pouch and leukocyte migration

Air pouches were raised on the dorsum of BALB/c mice as described elsewhere (Edwards et al., 1981). Briefly, on days 0 and 4, mice received a subcutaneous injection of approximately 2 ml sterile air using a 27G needle attached to a 0.2 μ syringe filter. On day 7, mice were injected with 1 ml PBS containing 50–100 μg r*Na*-ASP-2 or r*Ac*-MTP-1 in the air pouch. The dose of antigen used and timing of sacrifice for each experiment are indicated within each figure legends. After sacrifice, the pouches were flushed with 3 ml PBS to collect their contents. Cells were then filtered through a 70-μ cell strainer (BD Falcon, San Jose, CA), centrifuged and resuspended in 1 ml PBS. Leukocyte numbers were calculated and up to 2.5×10^5 cells were centrifuged onto glass slides by cytospin. Slides were stained with Wright–Giemsa (Camco Chemicals, Florence, KY) to identify leukocyte subpopulations. A total of 300 cells were counted for each slide. Individual leukocyte numbers were combined and expressed as mean ± SEM. Total cell numbers were obtained as the product of total cell number and percentages for each cell type.

2.4. Cell purification from peripheral blood

One to two milliliter of peripheral blood was obtained from the mice by cardiac puncture and treated with ammo-