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September 24, 2018

Andrea Weiser, Ph.D.
Archaeologist
Seattle City Light
500 Newhalem St.
Rockpoint WA 98283

Re: Results of Protein Residue Analysis for 1 artifact from site 45WH957, Whatcom County, Washington
AINW Report No: 4111

Dear Dr. Weiser:

At your request, Archaeological Investigations Northwest, Inc. (AINW), analyzed one projectile point from site 45WH957. The analysis was done to identify possible protein residues using cross-over immunoelectrophoresis (CIEP). There was one positive reaction to one of the eight chosen antisera.

The CIEP technique has been widely used in forensic laboratories to determine the origin of bloodstains as evidence in criminal investigations, and has been adapted for use in archaeology to detect protein residues on prehistoric artifacts. The CIEP technique is based on the immune (antigen-antibody) reaction. Extracts of protein residues from artifacts in an ammonia solution are tested against antisera from known animals. The solutions are placed on a gel substrate and exposed to an electric current which causes the proteins to flow together. An immune reaction between the extract and the antiserum causes a precipitate to form, which is visible after being stained.

The CIEP tests were conducted between September 17 and 23, 2018, by laboratory director Dr. Cam Walker. Extracts from the artifact were tested against bear, bovine, cat, deer, dog, goat, sheep, and trout antisera. The bear antiserum was custom-produced for AINW by Triple J Farms. The bovine, cat, dog, and goat antisera were manufactured by MP Biomedicals, LLC. The sheep antiserum was produced by Sigma-Aldrich Co., LLC. The deer antiserum was made by Bethyl Laboratories, Inc. The trout antiserum was custom-produced for AINW by Cocalico Biologicals, Inc. A chart included with this report shows the antisera that were used in the tests and the species found to react with each antiserum.

Standard analysis procedures began by extracting residue from the projectile point. The extracts were then placed singly into gels, and tested against the eight antisera with the CIEP technique. In addition to the artifact extract, positive and negative control sera were run with each gel. This was done to determine if there were any contaminants or extraneous proteins that may give false positive results. If an anomalous result such as an extract reacting with a negative control serum is obtained, the extract solution is mixed with an equal volume of a 1% solution of a non-ionic detergent to increase chemical bonding specificity, and is run through the CIEP process again. If a reaction still occurs after the addition of the non-ionic detergent, any reactions of those specimens to the antiserum are discounted. The extract analyzed for this project did not react with the negative control.

There was a single positive response to the goat antiserum. The goat antiserum reacts to proteins from Subfamilies Bovinae and Caprinae. In this instance, as specific tests were also conducted to narrow the results, ruling out bovine, deer, and sheep proteins, the result is most likely a positive to the presence of goat protein. The positive result was confirmed by repeat analysis. The results of the tests are indicated on the attached chart.

The results from testing against the selected antisera do not preclude the possibility of the artifact extract retaining residue from other animals. The liquid extract obtained from the artifact has been frozen for storage and will be retained for one year should you wish any additional tests. Please call or email us if you have any questions about the analysis or this report.

Sincerely,

John L. Fagan, Ph.D., R.P.A.
President/Senior Archaeologist

Cam Walker, Ph.D.
Supervising Archaeologist/
Laboratory Director

Attachments

ARCHAEOLOGICAL INVESTIGATIONS NORTHWEST, INC.

PROTEIN RESIDUE ANALYSIS COMPARATIVE RESULTS

Project: Protein residue analysis of one artifact from Site 45WH957

				TYPE OF ANTISERUM							
RAL #	SITE	CATALOG #	MATERIAL/ TOOL TYPE	Bear	Bovine	Cat	Deer	Dog	Goat	Sheep	Trout
1	45WH957	NOCA39513	Projectile point	-	-	-	-	-	+	-	-
GEL #				2749-1	2749-1	2749-1	2749-1	2749-2	2749-2	2749-2	2749-2
GEL #											

Key: + = Positive; - = Negative

Archaeological Investigations Northwest, Inc.

AINW RESIDUE ANALYSIS LABORATORY
ANTISERUM CHART

COMPANY	ANTISERUM	HOST	REACTS WITH
BLI*	BOTTLENOSE DOLPHIN	rabbit	Family Delphinidae: dolphins, less strongly with porpoises and toothed whales
	DEER	rabbit	Family Cervidae: white-tail and mule deer, elk, moose, caribou
	FERRET	rabbit	Family Mustelidae: ferret, otter, badger, mink, stoat, wolverine, marten
	WHITE WHALE	rabbit	Family Monodontidae: belugas and narwhals, porpoises
BYT*	DUCK	rabbit	Family Anatidae: swans, geese and ducks
CBI*	TROUT	rabbit	Subfamily Salmoninae: salmon, steelhead, rainbow trout, char
MP*	BOVINE	rabbit	Family Bovidae: domestic cow, bison
	CAT	goat	Family Felidae: cat, mountain lion, lynx, bobcat
	CHICKEN	goat	Order Galliformes, Order Anseriformes, Order Columbiformes
	DOG	rabbit	Family Canidae: domestic dog, coyote, wolf, fox
	GOAT	rabbit	Bovid Subfamilies Bovinae and Caprinae, less strongly with cervids
	GUINEA PIG	goat	Order Rodentia: guinea pig, porcupine, beaver
	HORSE	goat	Family Equidae: horse, donkey, mule, extinct equids
	MOUSE	goat	Order Rodentia: mice, rats
	RABBIT	goat	Family Leporidae: rabbit, jackrabbit
	RAT	goat	Order Rodentia: rats, mice, squirrels
NIL*	PIGEON	rabbit	Order Columbiformes: pigeons, doves
SIGMA*	HUMAN	goat	Order Primates: humans, apes, monkeys
	SHEEP	rabbit	Genus Ovis: domestic sheep, bighorn sheep
Triple J Farms (custom)	BEAR	goat	Family Ursidae: black bear, brown bear, grizzly
	CAMEL	goat	Order Artiodactyla: camelids, bovids, cervids, antilocaprids
	RHINO	goat	Family Rhinocerotidae: white, black, Indian, Javan, Sumatran, and extinct rhinoceros
	ASIAN ELEPHANT	goat	Asian and African elephants and extant and extinct members of Order Proboscidea

*Notes: BLI = Bethyl Laboratories, Inc., BYT = Biorbyt Laboratories, Inc., CBI = Cocalico Biologicals, Inc., MP = MP Biomedicals, LLC, NIL = Nordic Immunological Laboratories, Sigma = Sigma-Aldrich Co., LLC.

AINW RESIDUE ANALYSIS LABORATORY METHODS AND PROCEDURES

Blood protein residue analysis performed at the Archaeological Investigations Northwest, Inc. (AINW) Residue Analysis Laboratory uses the technique of cross-over immuno-electrophoresis (CIEP) to analyze protein residues extracted from the surface of stone artifacts and other objects. This technique has been widely used in forensic laboratories to determine the origin of bloodstains as evidence in criminal investigations, and has fairly recently been adapted for use in archaeology to detect protein residues on stone tools. The CIEP method used by the AINW Residue Analysis Laboratory is based on techniques developed by the Royal Canadian Mounted Police Serology Laboratory in Toronto, Ontario (Culliford 1971; Newman 1990; Williams 1990). The CIEP technique uses the immune (antibody-antigen) reaction, the principle that all animals produce immunoglobulin proteins (antibodies) that recognize and bind with foreign proteins (antigens) as part of the body's defense system. The ability of antibodies to precipitate antigens out of solution is the basis of CIEP analysis (Newman 1990:56). CIEP indicates the presence or absence of a particular antigen, and is not designed as a quantitative test. While other types of immunoassay have been used effectively to analyze blood protein residues under various conditions, the CIEP test is particularly suitable in that it is sensitive (able to detect protein in concentrations of about two parts per million), does not require expensive or bulky equipment, is relatively fast (about 48 hours per test), and can easily and efficiently accommodate multiple samples (Newman 1990:52).

BLOOD PROTEIN RESIDUES

Blood is composed of red and white blood cells and serum, which is composed of about 150 different proteins including albumin, alpha, and beta globulins. Immunoglobulins are large, Y-shaped proteins with antigen binding sites located on the V portion of the Y. There are several immunoglobulin molecules of different weight, size, and function. The most common type (and the most pertinent for CIEP) is immunoglobulin G (IgG). Other less common varieties are immunogobulin A (IgA), immunoglobulin D (IgD), immunoglobulin E (IgE), and immunoglobulin M (IgM). Some of these proteins can survive in the environment in a nonfunctional but immunologically identifiable form for long periods of time by forming a "covalently cross-linked proteinaceous mass with a high molecular weight" (Marlar et al. 1995:30). This combination of protein, fatty tissues and soil particles is resistant to microbes and is markedly insoluble in water. It seems probable that porosity and surface roughness of the artifact also aids in the preservation of protein residues. Experiments by AINW and others have identified blood residues from mammoth, bison, musk ox, horse, caribou, bear, duck, and trout on Paleoindian artifacts that may be as old as 11,500 years (Forgeng 1998; Loy and Dixon 1998; Williams 1993). Other studies suggest that protein residues can survive in recognizable form for as long as 40,000 years (Prager et al. 1980).

Artifacts can be examined under a binocular microscope (at around 240 x maximum magnification) to identify probable residues, as well as cells, hair and other tissues. Microscopic examination is not always effective as a screening technique as CIEP can still detect otherwise invisible residues. A common medical test for occult blood is sometimes effective when used to screen the extracted residue solution. However, the CEIP technique can detect residues in more dilute concentrations than is possible with the commonly available occult blood test.

THE IMMUNE REACTION

Immunological forensic tests owe their effectiveness to the antigen-antibody reaction, which allows very specific recognition and identification. Essentially, any molecule that can bind to an antibody is an antigen. For archaeological purposes, the antigen is an unknown protein adhering to an artifact after its use. Antigens are foreign proteins that, when introduced into the blood stream of an animal, stimulate the immune system of the animal to produce antibodies (most commonly IgG protein molecules) with specific binding sites that match corresponding sites on the foreign antigen. Polyclonal antibodies, which bind to multiple sites on the antigen and therefore have a high rate of successful matching to unknown proteins, are the most commonly used reactants in CIEP. The meeting of antigen and antibody forms a very strong bond between the two proteins. The visible line formed in a positive CIEP reaction occurs when an antigen with multiple binding sites matches a group of polyclonal antibodies, binds with them, and causes the proteins to precipitate out of solution (Marlar et al. 1995:28).

ANTISERA

The antisera used in AINW's CIEP analysis are obtained from commercial laboratories. A forensic antiserum is made by injecting a host animal, typically a goat or rabbit, with a protein solution obtained from another animal. The immune system of the host animal produces antibodies (mainly IgG) in reaction to the foreign antigen. Blood serum drawn from the host animal is purified and tested to determine the range of reactivity of the antiserum. The purified antiserum is then freeze-dried for storage and shipment. After receipt of a new lot of antiserum, the AINW laboratory routinely tests each antiserum against representative specimens from up to 32 different animal species.

THE AINW RESIDUE ANALYSIS LABORATORY

Ancient protein residues are often difficult to extract from the artifacts that have preserved them. The AINW Residue Analysis Laboratory uses a 5% ammonia solution, which has been used for similar applications in forensic medicine (Dorrill and Whitehead 1979; Kind and Cleevely 1969). Ammonia is generally more effective in lifting old and partially denatured blood proteins than other solvents (Newman 1990). A small amount of the ammonia solution is applied to the artifact in a plastic tray, and the tray and artifact are placed in an ultrasonic bath (Branson 2200) for 30 minutes or longer. The artifact in solution is then placed on a mechanical rotator (Thermolyne Rotomix) for an additional ten minutes. Artifacts too large for the ultrasonic extraction may be placed on the rotator for 30 minutes or longer. Residues from soil samples can also be extracted using variations of these methods. The extraction solution is then drawn off and stored in an airtight microcentrifuge tube. The extracts are centrifuged to clarify the sample, refrigerated, and the CIEP test is run as soon as possible after extraction. The extracts may be frozen immediately if testing is to be delayed for more than one week.

AINW's CIEP method uses an agarose gel as a substrate. Standard analysis procedures begin with extracting residues from the artifacts with a 5% ammonia solution. The artifact extracts are then placed singly into gels, and tested against the antisera selected for these tests with the CIEP technique. In addition to the artifact extracts, positive and negative control sera are run with each gel. This is done to determine if there are any contaminants or extraneous proteins that may give false positive results. If an

anomalous result such as an extract reacting with multiple antisera or to a negative control serum is obtained, the extract solution is mixed with an equal volume of a 1% solution of a non-ionic detergent to increase chemical bonding specificity, and is run through the CIEP process again. If a reaction still occurs after the addition of the non-ionic detergent, any reactions of those specimens to the antisera are discounted. Experiments at AINW have implicated plant pitch used in hafting prehistoric stone tools as a possible cause of some cross or non-specific reactions.

Electrophoresis is used to drive the antigens and antibodies together. The gel substrates are placed in acrylic electrophoresis tanks filled with barbital buffer solution, then attached to the regulated H.V. power source. The antibodies move toward the cathode because of the overall negative charge on the molecule, while the antigens move toward the anode. A precipitate is formed where the proteins meet and bond in the area between the wells, visible as a white line or arc (Culliford 1971). The gel is soaked overnight in saline to stabilize the reaction, then dried and stained with a standard protein stain as a permanent record of the CIEP results. The dried and stained gel is then backlit on a light table, and examined under magnification for the presence of precipitate lines, indicating positive reactions. After testing, the extracts are frozen and stored for one year in case additional testing is requested.

HINTS FOR ARTIFACT COLLECTION AND TREATMENT FOR RESIDUE ANALYSIS

For optimum results, the following suggestions are provided for archaeologists considering submitting artifacts for blood residue analysis (see also Marlar et al. 1995:36)

1. Handle artifacts as little as possible in the field. Avoid contamination by using latex gloves, the tip of a clean trowel, or other careful methods similar to the treatment of radiocarbon samples.
2. Do not brush off, spit clean, or wash the artifact. Since proteins are known to bind to soil particles, loss of adhering dirt may result in loss of blood antigen.
3. Place the artifact in a clean ziplock bag with as little loose dirt as possible.
4. Submit a small amount (about one tablespoon) of soil from the area adjacent to the artifact. As bacteria or animal excreta in the soil may cause false positive reactions, soil controls are useful for cross checking results from artifacts.
5. Positive results have been obtained from projectile points, scrapers, flake tools, debitage, bone, burned bone, fire-cracked rock, cobble tools, ground stone tools, and soil samples from features and general site contexts. Surface artifacts are also good candidates for residue preservation. Obsidian, CCS, and basalt artifacts are equally likely to preserve residues, although some more porous materials may contain more proteins.
6. When selecting the type of antisera for analysis, consider allowing for a broad range of testing supplemented by more specific testing of positive results (for example, testing positives for chicken against duck and pigeon to narrow the results). If an artifact tests negative for all of the selected antisera, it may still contain preserved residues from other species.

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