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BLOOD RESIDUES ON ARCHAEOLOGICAL OBJECTS — A CONSERVATION PERSPECTIVE

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ABSTRACT

The impact of human handling on studies of blood residues is demonstrated by experimental work undertaken on modern stone tools. The results are examined within the context of excavation techniques and conservation treatments that may influence laboratory analyses, with special emphasis on the consequences of washing and handling stone artifacts. The results of experimental work are compared with those obtained from a large assemblage of stone tools that was excavated along the shore of the Anacostia River in Washington DC. Because the amounts of biomolecules found on excavated materials are small, more sensitive biochemical techniques are being applied to archaeological artifacts. The implications of organic residue analysis for archaeologists, conservators and collections managers are considered, with emphasis on appropriate methods of excavation, treatment procedures and materials for storage. This paper focuses on the issues of immediate concern to conservators, and the impact that washing and handling may have on residue analysis.

INTRODUCTION

Experimental work on residues on stone tools is a continuing and somewhat controversial area of study. Considerable amounts of public money are being spent to identify sources of blood on tools, as if analytical approaches were well established and widely accepted. Given the extremely low levels of protein that were found in three studies on ancient and experimental tools [1-3], the authors chose to examine the apparently benign interventions of washing and human handling on subsequent blood residue testing.

Post-excavation interference with ancient residues and the dangers of contamination are important considerations in the curation and care of ancient artifacts. In the museum environment, the burden of care for objects and, ultimately, for the information that the objects hold lies with conservators. The conservator is responsible not only for the objects but also for residues associated with archaeologically recovered materials such as pottery and lithics.

Over the last decade, there has been mounting evidence to suggest that plant and animal residues remain closely bound to the surfaces of prehistoric stone tools. The extent of residue preservation, spatially, temporally, quantitatively and qualitatively, on archaeologically-derived stone tools is an area of current research [4-12]. The potential of blood residues in assessing tool use and paleodiet, as well as in genetic and taxonomic studies of evolution, justifies this developmental effort. Of particular interest is the contribution residues might make in cases where the small quantity of macro-organic remains has hampered interpretation.

There are many caveats in regard to blood residue analysis. Two research groups failed to detect blood after replica tools made from local rock were used to butcher a goat at an East African archaeological site, and were then subjected to immunological testing [2]. Laboratory exposure to ultraviolet irradiation, in order to test the sensitivity to sunlight of residues from butchery, resulted in the destruction of blood that had been detected by immunological methods on these experimental tools. Many researchers have discussed disagreements in the results of residue experiments, and controversy surrounds the mechanisms involved in both protein degradation and its survival over time [1, 4, 5, 9, 13-17].

CONVENTIONAL APPROACHES TO LITHICS

The treatment of archaeologically recovered material, including lithics, between the site of excavation and the laboratory varies. Approaches will differ from site to site and from one specialist to another. In the case of lithics, two fundamental, apparently harmless actions — washing and handling — are carried out routinely and often are the main initial stages of post-excavation processing.

Washing

Following excavation, lithic material is washed routinely to remove adherent soil and to expose the worked surfaces. This process is generally performed in the field by immersion in a bath of water, aided by soft brushing [18]. Many view this step as an essential part of research into lithic microwear [19, 20]. However, some researchers involved in microwear studies stipulate the use of more aggressive chemical cleaning solutions to remove concretions from the surface of lithics. In addition to detergents, acidic and basic solutions have sometimes been used [19]. This kind of cleaning is undertaken for a number of reasons, for example, to provide illustrations and, in particular, to allow detailed study of the micromorphology of use/wear patterns.

There have been many blood residue studies that made use of washed tools or tools that had an unknown history of curation [10, 17, 21, 22]. While some researchers have reported blood on tools that have been washed, these results do not necessarily mean that routine curation procedures can be considered benign interventions [6, 17]. An experiment designed to test the effects of gentle water-washing on two types of stone demonstrated that large quantities of blood, both freshly applied and aged, could easily be removed [22]. The study concluded that there is nothing to suggest that washing artifacts will be anything but detrimental to the recovery of organic residues.

From the site at Head-Smashed-In Buffalo Run in south-west Alberta, Canada, 10 lithics from earlier excavations and 21 samples from current excavations [17] were analysed. Almost half (4 out of 10) of the museum pieces (which had been washed) and only one quarter (5 out of 21) of the freshly excavated lithics showed any immunological reactivity. Considering the results of experimental washing [22], it might be anticipated that freshly excavated material would exhibit a greater concentration of bloodstained tools than washed pieces in museum storage; in fact the opposite was observed. Human handling is one possibility that may account for these unusual results.

Handling

In general, lithics are considered robust enough for careful handling. A lithic artifact may be subjected to routine handling many times on site, from initial removal from the soil by the excavator to detailed examination. In addition, a spectacular find will generate considerable interest and will often be passed around by members of the excavation team. Obviously, there is considerable potential for protein and DNA to be deposited as a result of skin shed onto the surface of archaeological lithics.

A number of residue studies in recent years have involved museum collections. Microscopic contaminants were observed on material excavated from Tabun Cave, Israel, in the early 1930s: synthetic fibres from clothing and fragments of cellulosic material were found adhering to the surface of several lithics [10]. These 'artefacts' could only have been introduced 'during curation'. There has also been the question as to whether human handling could introduce sufficiently high levels of protein to confuse the results of blood residue analysis.

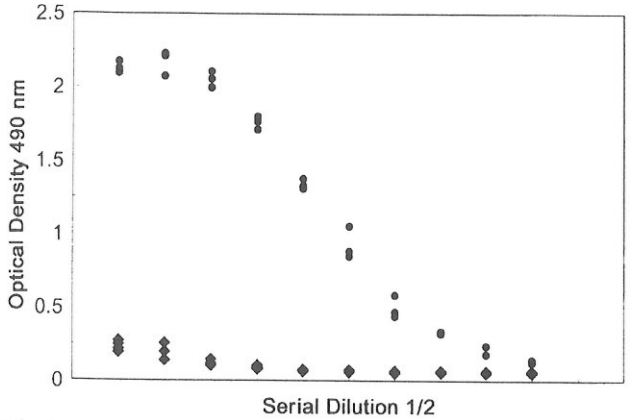

Fig. 1a The reactivity of the *handled* lithics (diamond) relative to the lithics *bloodied* with fresh goat blood (circle), using a rabbit anti-goat whole serum polyclonal antibody (concentration 1:1000) in a serially diluted (0.5) ELISA assay.

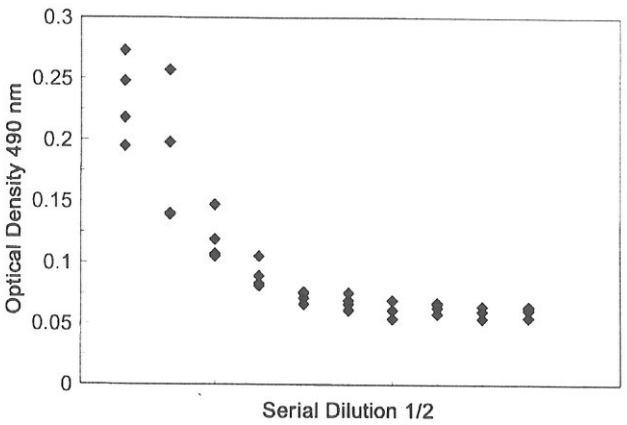

Fig. 1b Reactivity of the serially diluted *handled* lithics with rabbit anti-goat whole serum polyclonal antibody (concentration 1:1000). Average blank value is 0.056 (n = 20).

MATERIALS AND METHODS

In order to investigate the impact of human handling on lithic material, eight small replica projectile points (arrowheads) were purchased at a local nature products store and subjected to testing. These eight experimental tools, made of two rock types (obsidian and a chert-like material), were handled by members of staff at the Conservation Analytical Laboratory. Goat blood was then applied to four of the experimental tools (two obsidian and two chert) and allowed to dry. All eight experimental lithics were extracted in a solution of guanidine isothiocyanate to remove protein residues (see Appendix).

The identification of degraded molecules is best achieved using a range of techniques that optimize sensitivity and are also selective. A combination of immunochemical techniques, enzyme-linked immunosorbent assay (ELISA) and Western blotting [23, 24], was used to complement SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). Following initial testing by ELISA, the extracts were desalted using a porous polyacrylamide gel (P6-DG) and concentrated further by freeze-drying. Samples were rehydrated in deionized water. Details of experimental procedures may be found in the Appendix.

The proteins recovered from the experimental tools were compared to those retrieved from archaeological lithics uncovered during excavations carried out before highway construction. The four sites making up the Barney Circle Freeway Improvement Project on the Anacostia River typified a major application of blood residue analysis. These were publicly funded but privately excavated projects, conducted on publicly-owned land. The proximity of the sites to the Conservation Analytical Laboratory allowed some control over sampling and excavation, and therefore provided a certain degree of control over lithic recovery.

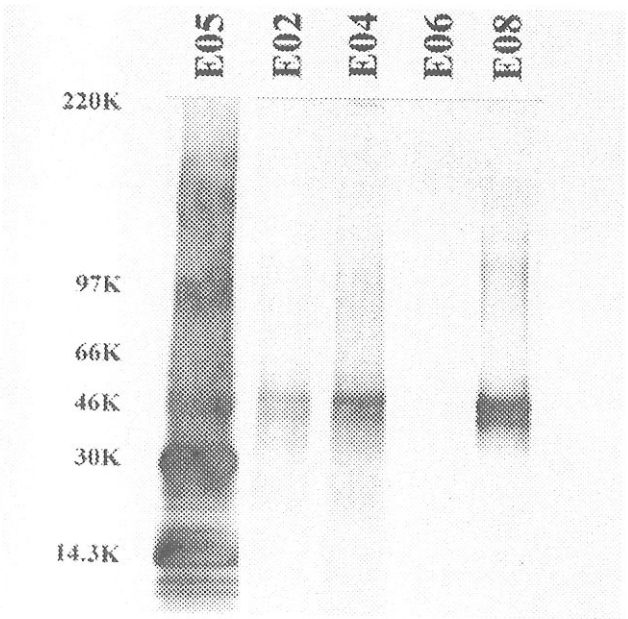

Fig. 2 Following gel electrophoresis and electrophoretic transfer to nitrocellulose, three out of four *handled* experimental tools (E02, E04, E08) show demonstrable levels of protein after gold staining. The keratin doublet is seen clearly in all three extracts. A single lane of *bloodied* extract (E05) is shown for comparison. Multiple banding shows the presence of many proteins.

RESULTS

In order to determine whether human handling had any effect on immunological analysis, extracts from bloodied tools were compared to extracts from blood-free, handled tools. Modern goat blood can be removed from the surface of lithic materials using guanidine thiocyanate (Fig. 1a), by monitoring the binding of a polyclonal antibody (rabbit anti-goat whole serum at a concentration of 1:1000) in an ELISA assay. Extracts from the blood-free/handled experimental lithics were serially diluted with guanidine thiocyanate, 1:2, across consecutive wells of a microtitre plate. This sequential dilution of extract is a commonly used procedure to test the ability of the extract in question to 'dilute out'; that is, binding of the test antibody should become less as the test extract becomes more dilute. The handled tools were four to five times more reactive than the blank control lanes to a whole serum antibody (Fig. 1b). Therefore, handled tools without any blood on them give false positives for blood serum. The same tools were washed, heated to 400°C and re-extracted. Following this treatment the experimental tool extracts were identical to the background values. The immunological response illustrated in Figure 1b was derived from protein extracted from the surface of the experimental lithics (that is, from human handling) and was not a function of non-specific object cross-reactivity. Handling has the net effect of raising the background.

The source of this cross-reactivity in the handled tools was determined by visualizing the extracts after gel electrophoresis (Fig. 2). Three out of the four blood-free/handled tools exhibited demonstrable levels of protein. The protein was α-keratin (the major protein in skin), based on molecular weight determination by gel electrophoresis. It is not clear why one tool should have escaped contamination by skin-derived protein.

The cross-reactivity of one of the handled tool extracts to a whole serum antibody was confirmed by Western blotting

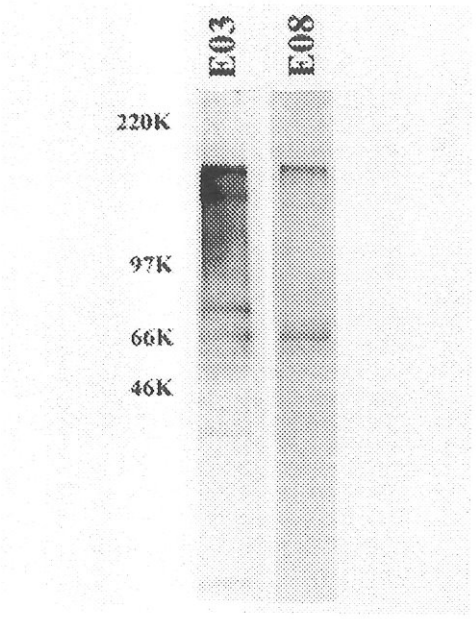

Fig. 3 Western blot using rabbit anti-goat whole serum antibody (1:500) in PBS-NFDM as first antibody. A bloodied extract (E03) is compared with a non-bloodied, handled extract. In addition to the keratin doublet, further reactivity is seen at higher molecular weight, suggesting the presence of more than one protein on the non-bloodied, handled lithics.

(Fig. 3). The bloodied tool extract (E03) reacted to this whole serum antibody at several separate and discrete sites. The blood-free/handled tool extract (E08) cross-reacted to the same antibody at two discrete molecular weights. It was not determined in these experiments whether this cross-reactivity on the handled tools is due to interference by skin proteins, represents blood from a cut sustained during handling (no injuries were reported), or derives from secreted serum proteins. The net effect is the same: handling can compromise immunological analyses of ancient tool extracts and should be avoided.

One critical question — can human handling confound and mimic ancient proteins — is addressed in Figure 4. Positives were defined as those extracts which exhibited at least twice the reactivity to a given antiserum when compared to a soil extract control. The protein was, however, present in minute quantities. Comparison of the archaeological values with those from the blood-free/handled lithics illustrates that there is real potential for confusion with ancient samples. Many of the extracts from the Late Archaic/Early Woodland site of Barney Circle did show evidence of blood when measured by ELISA assay. However, the extent of the antibody binding was, in many cases, in the range observed using the same antibody for experimental, handled tool extracts. In this experiment these handled lithics are the appropriate 'blank', not the soil or the chemical reagents in the analysis, both of which exhibited lower backgrounds in the assay.

STANDARDS FOR EXCAVATION, HANDLING AND CURATION

If residue analysis is desired, removal of lithics from the ground should be done using unpowdered disposable latex or vinyl gloves. Any adhering soil should be retained and not brushed or washed off. Soil samples should be collected from the same context (as well as a number of soil samples from around the site to be treated as controls). Lithic samples should be bagged individually in sterile polyethylene bags, and clearly labeled with contextual information and a warning to ensure that they are only handled while wearing gloves. Re-usable cotton gloves [25] are

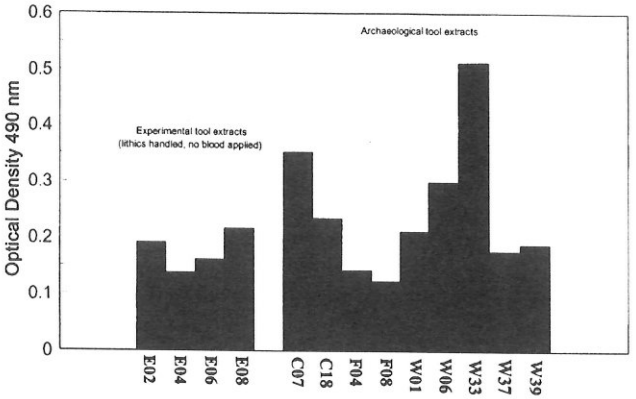

Fig. 4 Comparison of values for the *handled* lithics with lithics excavated at Barney Circle (after subtracting average blank values for both) indicates little difference in reactivity.

inadequate protection. Following excavation, material should be stored at 4°C as soon as possible, to avoid protein breakdown and microbiological activity.

Routine procedures such as washing and handling will compromise residue analysis, although water washing may not necessarily remove all of the adherent organic residue. Lithics destined for residue analysis should be left unwashed and stored carefully in cool, dry conditions away from light, in such a way as to avoid damaging one another. Detailed records should be kept of curated lithics and handling, beyond initial examination and study, must be carefully controlled.

After excavation and study, most lithics are destined for storage. Collections already in storage vary greatly in terms of curation standards. Often, access to the material is unrestricted and, since stone is a robust material, little care is taken in storage or handling. Manipulation of tools, such as re-attaching flakes with adhesive or applying a coating for a catalogue label, should be undertaken only with the full understanding that future biochemical study will be compromised. Adhesives and consolidants will often render materials unsuitable for further study [26].

Although many researchers assume that proteins are robust materials that can survive harsh conditions outside the body, while still retaining their antigenicity and biological activity [27], optimum conditions should be maintained in order to ensure the success of biochemical analysis. The research value of ancient biomolecules is best considered before an excavation starts and the strategy for collecting material should reflect this concern.

DISCUSSION AND CONCLUSIONS

Many cases of site conditions which favour biomolecular preservation have been reported. Increasingly, however, protein and DNA survival after excavation is an important consideration. The most direct threats to blood residues that concern the conservator are: (1) the excavation and post-excavation handling and treatment of artifacts and (2) the conservation and curation of lithic material in museums and in storage facilities.

Contamination is of serious concern to those working with ancient biomolecules. The sensitivity of immunological testing and polymerase chain reaction (PCR) based amplification techniques for DNA analysis requires stringent control. Re-testing samples simply to rule out modern human contamination is costly in terms of time and effort. From the point of view of the laboratory, it is wise to work only on excavated material which comes directly to the laboratory immediately after excavation. These criteria would tend to exclude archaeological material which has been excavated following current practice, and all material housed in collections under present standards.

Human handling can result in false positives from immunological analysis of ancient blood residues. The results of the handling experiments described here indicated substantial transfer of skin protein.

All antibodies used in this study produced some reaction with the blood-free/handled lithics in the ELISA experiments, highlighting a major shortcoming in blood residue work: when concentrated amounts of polyclonal antisera are necessary for analysis, as in cross-over immuno-electrophoresis. Therefore, the results of blood residue analysis should not be trusted if the specimens have been handled previously and proper immunological controls have not been applied. It should be possible to screen antisera used in blood residue work and select only those which show no 'handling' cross-reactivity, at appropriate concentrations.

Ancient proteins bound to the surface of lithics are usually present in minute quantities, and appropriate measures must be taken during the initial excavation, treatment and storage to ensure later successful molecular analysis. Because it is rare for specialists to be present on site, the burden of care for lithic material and residues usually falls on the excavator, who must be informed of the risks so as to minimize problems of contamination and damage.

APPENDIX

Extraction protocol

Both the experimental lithics and 88 archaeological lithic samples (flakes, worked stone and 'tools'), from sites 51SE26 and 51SE31 at Barney Circle, were extracted using the same procedure. Each lithic was placed in a sterile sample bag and covered by a minimum volume of the guanidine isothiocyanate solution: 4M guanidine isothiocyanate, 0.02M sodium acetate (pH 5.2), 0.0001M dithiothreitol, 0.5% N-lauryl sarcosine. The samples were extracted in a sonic bath for a total period of one hour each, with five-minute intervals to guard against heat build-up. The samples were then left over night on a vibrating table before storage at 4°C. The extract in each case was passed through 0.45µm filters before use. The archaeological material was washed thoroughly in a succession of water baths. Guanidine thiocyanate solution was found to be an effective extracting solution for the removal of blood from the surface of lithics, and albumin left for five weeks at room temperature in this solution exhibited no obvious signs of degradation as determined by gel electrophoresis.

Enzyme-linked immunosorbent assay (ELISA)

ELISA assays measure the amount of antibody bound to an antigen, in this case blood removed from the experimental tools. The test is carried out in small plastic wells, and the amount of bound antibody is determined by measuring the colour development of a reagent that has been conjugated to an antibody. The results are quantitative if performed in the linear range of the assay, and can be obtained with both small volumes of extracting solution and small amounts of antigen (for example, blood).

Extracts from the experimental 'handled' lithics were diluted serially (1:2) and incubated overnight at 4°C. ELISA assays were carried out according to the methods of Fisher *et al.* [28] and Tuross and Dillehay [3]. The blocking reagent was 0.5% non-fat dried milk in phosphate-buffered saline (PBS-NFDM). Specific antisera used are noted in 'Results', and were diluted in PBS-NFDM and passed through 0.45µm filters before use.

Colour developer, *o*-phenylenediamine dihydrochloride (OPD), was prepared immediately prior to use. Development was stopped by the addition of dilute sulphuric acid to each well. ELISA plates were examined at a wavelength of 490nm.

Gel electrophoresis (SDS-PAGE)

Desalted extracts were rehydrated in 100µl of deionized water. Equal volumes of sample and 2× SDS sample buffer were heated for five minutes at 100°C. The samples were run on 4-20% tris-glycine polyacrylamide gels. The gels were run at constant current (15mA per gel) and the voltage set not to exceed 250V.

Transfer to nitrocellulose (0.45µm pore size) was carried out at 100V for two blots (about 10 minutes each). The nitrocellulose was washed in phosphate-buffered saline with a final wash in deionized water to remove all traces of the SDS from the buffer solution. The blots were stained overnight in colloidal gold.

Western blotting

Western blotting employs the same antigen/antibody binding approach as used in ELISA assays, with the added dimension of molecular weight determination for the extracted materials. The antigen, in this case blood, is subjected to gel electrophoresis, thus separating proteins on the basis of molecular weight on a solid support (the gel). After electrophoresis, the proteins are moved under constant voltage from the gel to a piece of nitrocellulose, keeping the spatial register of the separated proteins. The nitrocellulose membrane is then soaked in antibodies and a colour reaction documents where the antibody binds to the membrane, giving the molecular weight of the reacting protein.

After gel electrophoresis and transfer to nitrocellulose, Western blotting was performed following established procedures [3, 29].

Rapid desalting

Disposable pipette columns (10ml) were filled with hydrated gel suspension (P6-DG: exclusion limit 6000 daltons) and rinsed with 0.05M ammonium acetate (titrated to pH 7.2). Aliquots of extract (2ml) were placed on individual columns and eluted with ammonium acetate. The desalted extracts were frozen at –20°C and freeze-dried over night.

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