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Analysis of Restriction Fragment Length Polymorphisms in Deoxyribonucleic Acid (DNA) Recovered from Dried Bloodstains

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ABSTRACT: Deoxyribonucleic acid (DNA) was recovered from dried bloodstains aged up to three years and shown to be of high molecular weight. DNA was digested with restriction endonucleases and fractionated by agarose gel electrophoresis. Following transfer to a filter, DNA was hybridized with two different radioactively labeled recombinant probes which recognize highly polymorphic regions in human DNA. The autoradiographic pattern observed was not altered by sample age, and the size of the alleles was consistent with those observed in the general population. Therefore, DNA of high molecular weight prepared from dried blood samples can be used for identification.

KEYWORDS: pathology and biology, blood, genetic typing, deoxyribonucleic acid (DNA)

Biological materials recovered as evidence can be analyzed at the molecular level to help establish identity. Electrophoretic and immunologic techniques are currently used to type many polymorphic proteins and cellular antigens [*1,2*]. Blood evidence, however, usually enters the laboratory in the form of dried stains, and the drying, aging, and contamination associated with these stains limits the number of markers that can be typed. Although the potential for discrimination using protein markers is significant, it is desirable to have another type of marker that both survives better than proteins and possesses a larger number of distinguishable alleles.

Reliable methods are now available to detect polymorphisms in DNA, the genetic material that codes for all inherited differences. These methods rely on restriction endonucleases, enzymes that cleave DNA at sequence specific recognition sites, generating a reproducible pattern of fragments from an individual's DNA. A particular DNA restriction fragment can exhibit heritable polymorphisms with respect to size [*3,4*]. These restriction fragment length polymorphisms (RFLPs) can be visualized using DNA hybridization technology. This article presents methods for isolating high molecular weight DNA from dried bloodstains and for subsequent analysis using two probes that recognize highly polymorphic regions of human DNA. The experiments demonstrate the feasibility of matching the polymorphic patterns of

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DNA recovered from a bloodstain with the DNA patterns obtained from a blood sample drawn from a suspected individual.

Materials and Methods

Preparation of Dried Blood Samples

Samples were prepared to simulate bloodstain evidence found on clothing and on a smooth surface. Six 1-mL aliquots of freshly drawn blood were pipeted onto sections of a cotton shirt, producing stains approximately 45 mm in diameter. Another six aliquots were pipeted into 50-mL glass beakers, producing stains just covering the bottoms. These samples were allowed to age at room temperature up to 28 days before DNA was isolated. Three forensic blood samples taken postmortem were stored as dried stains in a cold room for two to three years. These stains were on heavy white cotton cloth and were also approximately 45 mm in diameter.

Isolation of DNA

Peripheral blood samples were mixed with four volumes of blood lysis buffer (0.32*M* sucrose, 10m*M* Tris hydrochloric acid pH 7.6, 5m*M* magnesium chloride, 1% Triton X 100). The white cells were pelleted by centrifugation at 1000 × *g* for 10 min at 4°C and resuspended in DNA lysis buffer (10m*M* Tris hydrochloric acid pH 7.4, 10m*M* sodium chloride, 10 m*M* ethylenediaminetetraacetate [EDTA]). Proteinase K and sodium dodecylsulfate (SDS) were added to final concentrations of 100 μg/mL and 1.0%, respectively. Samples were incubated at 37°C overnight on a rocker platform to allow gentle mixing. The DNA solutions were extracted twice with phenol/chloroform (1:1), twice with chloroform, and dialyzed against three changes of a thousandfold excess of TE buffer (TE: 10m*M* Tris hydrochloric acid, pH 7.4, 1m*M* EDTA). Ammonium acetate was added to a final concentration of 0.3*M* and the DNA was precipitated with 2.5 volumes of 95% ethanol overnight at −20°C. DNA was pelleted and resuspended in 1 mL of TE buffer. Bloodstained cotton cloth cut into small strips or dried blood scraped from glass beakers was suspended directly in DNA lysis buffer and treated as described above. DNA concentrations were determined by absorbance at 260 nm on a Gilford model 260 spectrophotometer.

Digestion and Electrophoresis

One to ten micrograms of DNA were digested with restriction endonucleases Eco R1 or Taq 1 (Bethesda Research Laboratories) according to the conditions recommended by the manufacturer. A sixfold excess of enzyme was used for the initial digestion. Samples were ethanol precipitated, resuspended in 20-μL volumes and redigested with an additional two- to three-fold excess of enzyme. Two microlitres of each sample were visualized on a test gel to confirm complete digestion. Digested DNAs were size fractionated by agarose gel electrophoresis in TAN buffer (40m*M* Tris hydrochloric acid, pH 7.9, 20m*M* sodium acetate, and 2m*M* EDTA). Eco R1 digested DNA was electrophoresed in 0.4% gels at 0.6 V/cm for three days, with recirculation of the buffer. Taq 1 digested DNA was electrophoresed in 1.2% gels overnight at 1.8 V/cm. Appropriate size markers, prepared by digesting DNA from lambda and ϕX174 bacteriophage with various restriction enzymes, were included in each gel. DNA was visualized by ethidium bromide staining and photographed under ultraviolet light. All these techniques can be carried out as described by Maniatis et al [*5*].

DNA Transfer and Hybridization

DNA was transferred to nylon membranes (Zetabind, AMF Cuno) using conventional blotting techniques [*5*]. DNA probes were radioactively labeled by nick translation [*6*] to

specific activities $>10^8$ cpm/μg with all four [α-^{32}P] deoxyribonucleotide triphosphates (Amersham). Membranes were hybridized with the labeled probes essentially as described by the manufacturer. Purified inserts from plasmids pAW101 and pLM0.8, isolated by the method of Vogelstein and Gillespie [7], were used as probes. Nonspecifically bound radioactivity was removed by washing the membranes in several successive changes of 2 × SSCP buffer (SSCP: 150m*M* sodium chloride, 15m*M* sodium citrate, 25m*M* $NaPO_4$), 1% SDS at 65°C in an ultrasonic cleaner, followed by a 0.4 × SSCP, 0.2% SDS wash, and a 0.1 × SSCP, 0.05% SDS wash, both at 65°C. Bands were visualized by autoradiography at -70°C using X-ray film (X-Omat, Kodak) and DuPont Lightning-Plus intensifying screens.

DNA Probes

The two DNA probes were propagated as cloned inserts in the plasmid pBR322. The plasmid pAW101 (kindly provided by R. White) contains a 6.5 kilobase (kb) Eco R1 insert that hybridizes to a region on chromosome 14 polymorphic with Eco R1 [8]. The plasmid pLM0.8 contains a 879 base pair Cla 1-Sph 1 insert derived from the flanking region of the Harvey ras oncogene on chromosome 11 [9]. The region is polymorphic with Taq 1.

Results and Discussion

The first parameter examined was the quantity of DNA recoverable from dried bloodstains. The amount isolated from 1-mL (45-mm) stains varied from 27 to 73 μg. The average recovery was approximately 40 μg, similar to the amount obtained from 1 mL of peripheral blood. Results using different amounts of DNA indicate that a signal on an autoradiogram can be generated with 1 to 4 μg (data not shown). Thus, a 200-μL bloodstain would be sufficient for an analysis using two probes.

The presence of high molecular weight DNA is essential for RFLP analysis. The detection of a fragment depends on its size-specific mobility in a gel. A visible band on an autoradiogram is obtained only if enough intact copies of the desired fragment are present. Broken fragments will migrate faster and will not be concentrated in a single band after electrophoresis. The approximate size of DNA recovered from bloodstains was determined by agarose gel electrophoresis with appropriate size markers (Fig. 1). Undigested DNA that ran slower than the largest marker fragment was considered to be of high molecular weight (>23 kb). High molecular weight DNA was obtained from all stain samples, including the two- and three-year-old specimens.

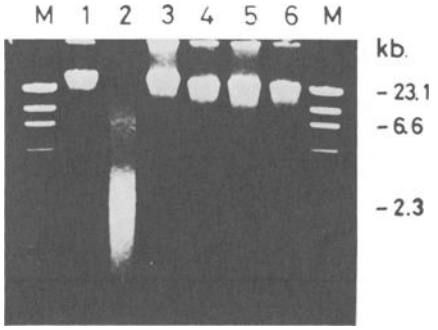

FIG. 1—*Isolation of high molecular weight DNA from bloodstains. Approximately 0.5 μg per lane of DNA was fractionated by electrophoresis in a 0.8% agarose gel containing ethidium bromide and photographed under ultraviolet light.* (1) *High molecular weight control;* (2) *degraded control;* (3-6) *DNA isolated from dried bloodstains three days, two years, two years, and three years old, respectively; and* (M) *Lambda-Hind III size markers.*

To visualize the RFLPs, DNA was digested with a particular restriction enzyme and size fractionated by electrophoresis in an agarose gel. The DNA was transferred to a filter membrane by a capillary blot procedure. A specific, purified sequence of DNA, obtained through molecular cloning techniques, was radioactively labeled and used as a probe. The probe was hybridized to the DNA on the filter, binding only to complementary DNA sequences. Autoradiography of the filter revealed one (homozygote) or two (heterozygote) allelic bands. Two DNA probes that hybridize highly polymorphic regions of DNA were used in this study, pAW101 and pLM0.8. The RFLPs recognized by both probes display Mendelian inheritance patterns [*8,10*]. Greater than 30 alleles can be distinguished for pAW101 with sizes ranging from 14 to 28 kb. At least 18 alleles have been observed for pLM0.8 with sizes ranging from 1.8 to 4.5 kb (unpublished results).

DNA isolated from stains on cotton and glass at timepoints of 0, 1, 3, 7, 14, and 28 days was subjected to blot hybridization analysis using the pAW101 and pLM0.8 probes. Clearly defined bands were seen on autoradiograms for all samples. Allele sizes were consistent and remained unchanged as the samples aged (Fig. 2). DNA from the two- and three-year-old stains was analyzed (Fig. 3) and produced bands within the size range of alleles observed in the general population.

Current technology allows the typing of a wide range of polymorphic protein markers in blood, including red cell enzymes, red cell antigens, and serum proteins. Only a fraction of these markers are sufficiently stable for use in bloodstain analysis. Of these, about 15 are commonly typed in the serology laboratory. Among those with the best discrimination probabilities are the phosphoglucomutase, erythrocyte acid phosphatase, and glyoxylase I isoenzymes; the immunoglobulin, group-specific component, and haptoglobin serum proteins; and the ABO, MNSs, and Rh antigen systems [*1,2*].

There have been several attempts to type bloodstains for human leukocyte (HLA) antigens, the most polymorphic protein system known [*11*]. The method, requiring intact lymphocytes and very highly characterized reagents, presents significant problems for dried stains, including cross-reactivity. Only a few HLA antigens have been typed successfully, and the system has not been widely accepted for bloodstain identification.

Protein analysis of old bloodstains is difficult because most markers suitable for typing persist for only a short time. Many of the proteins degrade within weeks, and the discrimination potential decreases accordingly. A study on the aging of proteins in bloodstains by De-

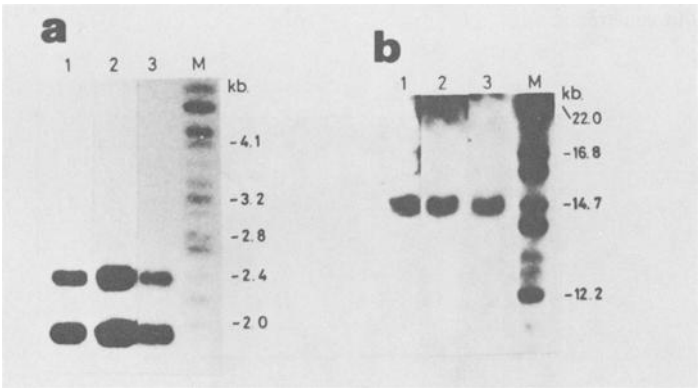

FIG. 2—*DNA analysis of dried bloodstains from a single individual.* (A) *Pattern of hybridization of pLM0.8 to Taq 1 digested DNA isolated from dried blood on glass:* (1) *fresh blood control;* (2) *one-day-old bloodstain;* (3) *twenty-eight-day-old bloodstain; and* (M) *size markers.* (B) *Pattern of hybridization of pAW101 to Eco R1 digested DNA isolated from dried blood on cotton:* (1) *fresh blood control;* (2) *three-day-old bloodstain;* (3) *fourteen-day-old bloodstain; and* (M) *size markers.*

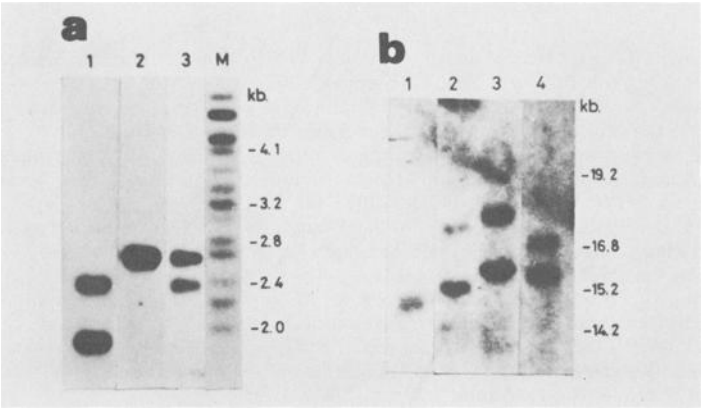

FIG. 3—*DNA analysis of old bloodstains from different individuals. Stains were on cotton.* (A) *Pattern of hybridization of pLM0.8 to Taq 1 digested DNA after* (1) *twenty-eight days.* (2) *two years, and* (3) *three years;* (M) *size markers.* (B) *Pattern of hybridization of pAW101 to Eco R1 digested DNA after* (1) *twenty-eight days.* (2) *two years.* (3) *two years, and* (4) *three years.*

nault et al [*12*], including six red cell antigen systems and four enzyme systems, calculates that the ability to discriminate between individuals decreases by two orders of magnitude between one week and twenty-six weeks of age. For the two- and three-year-old bloodstains used in this study most of the commonly analyzed proteins cannot be typed. DNA, which is chemically more stable than proteins, appears to persist significantly longer in dried bloodstains.

Another potential problem with protein analyses is the occurrence of false positives and false negatives. In antigenic reactions certain materials such as wool, nylon, and denim can create artifacts [*12*]. Changes occurring in proteins in the dried state can alter observed electrophoretic patterns [*1*]. As a bloodstain ages, such typing difficulties become more acute. Erroneous results may be avoided with DNA analysis because degraded DNA does not produce any bands on an autoradiograph. Incorrect bands, larger than expected, are obtained if the DNA of high molecular weight is not completely digested by the restriction enzyme. This situation is avoided by using an excess of enzyme and by redigesting the sample as described.

The power of an RFLP for identification is dependent on the number and frequency of the observed alleles. Analysis of hundreds of random individuals has generated a statistical data base of allele frequencies for the two probes discussed, which will be reported elsewhere. Work is progressing on additional probes that will further increase the power of a DNA-based identity determination. The system should be especially useful for old or contaminated bloodstains where protein determinations cannot be made. The conditions in this report are more controlled than those likely to be encountered in the field. Further work to investigate the effect of a variety of environmental factors on DNA recovery from bloodstains is in progress.

Conclusion

These experiments demonstrate that DNA of a quality sufficient for blot hybridization analysis can be recovered from dried bloodstains as much as three years old. For purposes of identification, analysis of DNA restriction fragment length polymorphisms should prove to be a powerful addition to current protein identification techniques.

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