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Development of a Radioimmunoassay Technique for the Detection of Human Hemoglobin in Dried Bloodstains

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ABSTRACT: A sensitive radioimmunoassay for the detection of human hemoglobin in dried bloodstains for the purpose of forensic science species identification has been developed. Bloodstains from 13 animal species were tested and found to be negative for human blood. A minimum volume of 0.8 μL of fresh blood is required to produce sufficient stain for successful testing. Bloodstains prepared from newborn and sickle-cell bloods were determined to be human. Bloodstains ranging in age from 1 month to 6 years which had been maintained desiccated at 20 to 25°C were also successfully tested. Positive results were obtained on human bloodstains stored at 24°C with relative humidity ranging from 0 to 98% for a period of 3 weeks. Absolute counts per minute (CPM) decreased with increased humidity. Human bloodstains exposed to bacterial contamination (gram positive or negative species) under humid conditions for 2 weeks also tested positive. Bacterial contamination caused a decrease in CPM, but insufficient to result in an erroneous conclusion as to species of origin. Positive results were also obtained on human bloodstains stored for 6 weeks at various temperatures ranging from −16 to 37°C. No significant decreases in CPM were noted for any of the temperature conditions described.

KEYWORDS: forensic science, blood, serology, radioimmunoassay, hemoglobin, bloodstains

Identification of a human bloodstain is generally conducted as a three-step procedure. First, presumptive tests for blood performed on the questioned stain must indicate that blood is present. Second, the stain is subjected to confirmatory testing to verify the presence of blood. Third, serologic methods are used to show that the stain is of human origin.

The existing methods to accomplish this task are usually easy to perform and are sensitive and specific, but misleading results occasionally can occur. The presence of bacterial contamination in a suspected stain may produce false positive results with presumptive catalytic tests. Interference by materials such as catalase, vegetable peroxidases, cytochromes, strong oxidizing agents, and metallic salts has been well documented [*1*]. Other methods, such as confirmatory microcrystalline tests, suffer from a lack of sensitivity. Analysts must be cautious in the interpretation of test results.

In an attempt to overcome these problems, a sensitive radioimmunoassay (RIA) technique for human hemoglobin has been developed. In the past, there have been few efforts to de-

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velop an assay of this type because of the limited availability of commercial high-titer antiserum against human hemoglobin [2]. Recently, however, such antiserum has become available.

Development of an RIA technique for human hemoglobin offers two distinct advantages. First, this technique, like all other hemoglobin assays using an anti-human hemoglobin antiserum, allows for simultaneous identification of a suspected stain as blood and as being of human origin. The conventional three-step process for the identification of a human bloodstain, which normally can take a day or longer, can be made in as little as 3 h using a one-step RIA technique. Second, RIA offers unparalleled sensitivity. In a clinical application, a radioimmunoassay technique was found to detect human hemoglobin when present in nanogram amounts [3]. An RIA technique for the detection of human beta-thromboglobulin in dried bloodstains was reported to be at least one order of magnitude more sensitive than conventional techniques for human bloodstain identification [4].

Materials and Methods

Determination of Species Specificity in Anti-Human Hemoglobin Antiserum

The specificity of the anti-human hemoglobin antiserum (Organon Teknika, Malvern, Pennsylvania) was determined using the Ouchterlony double immunodiffusion method [5]. The undiluted anti-human hemoglobin antiserum was run against human, chimpanzee, baboon, and rhesus monkey bloodstain extracts. Bloodstain extracts from nonhuman primates were chosen because the hemoglobins from these animals are structurally similar to that of normal human hemoglobin and thus provide a rigorous test of cross reactivity. A rabbit bloodstain extract and a substrate blank were also run against the anti-human hemoglobin antiserum.

Preparation of ^{14}Carbon-Labeled Anti-Human Hemoglobin Antibodies

Anti-human hemoglobin antibodies were radiolabeled with ^{14}Carbon by mixing 1.0 mL of anti-human hemoglobin antiserum with one vial of ^{14}C-formaldehyde (New England Nuclear, Wilmington, Delaware) in the presence of 20 μL 0.8*M* sodium borohydride and allowing the mixture to stand at room temperature for 20 min. One vial contains 0.0032 mL (aqueous solution) and 0.05 mCi of ^{14}C-formaldehyde having a specific activity of 47 mCi/mmole. Sodium borohydride causes binding of the antibodies with the ^{14}Carbon to occur. It does this by reductive amination involving the ϵ amino group of lysine residues in the polypeptide chains of the antibodies, that is, reduction of the Schiff base formed with ^{14}C-formaldehyde leads to methylation [6].

The ^{14}C-labeled anti-human hemoglobin antibodies were then isolated using molecular exclusion column chromatography [7]. Approximately 50 mL of column buffer containing swelled Sephadex G-50 Fine beads (Pharmacia Inc., Piscataway, New Jersey) was poured into a column (50 cm in length and having an internal diameter of 0.7 cm) and allowed to settle. Column buffer was prepared by dissolving 2.29-g Tris base, 3.72-g ethylenediaminetetraacetate (EDTA) disodium salt, 29.82-g potassium chloride, 0.5-mL thioglycerol, and 50-mL glycerol in 1.0 L distilled water and adjusted to pH 7.4 with 1*N* sodium hydroxide at 25°C. The mixture, containing radiolabeled antibody, was placed on the column and allowed to equilibrate for 20 min. Fractions consisting of 35 drops were collected, and 10 μL aliquots of each fraction were counted using a Packard Prias PI Tri-Carb liquid scintillation counter. A plot of counts per minute (CPM) (corrected for background) versus volume in millilitres of column eluate was constructed, revealing 2 well-separated peaks of radioactivity. The first peak represents the fraction of antibody bound radioactivity while the second represents the fraction of free (unbound) radioactivity. All fractions within the first peak

were pooled. This radiolabeled antibody preparation was used in all subsequent radioimmunoassay studies.

Hemoglobin Determination

A colorimetric method using Drabkin's reagent was used to determine total hemoglobin in six whole blood specimens spectrophotometrically. Hemoglobin is converted to cyanmethemoglobin. The concentration of the latter, which is proportional to the former, can be determined by monitoring the absorbance at 540 nm. A methemoglobin standard solution is used to construct the calibration curve (Sigma Diagnostic Kit No. 525, St. Louis, Missouri).

Radioimmunoassay for the Identification of Human Hemoglobin in Dried Stains: Procedure

The bloodstain was extracted in 1.0 mL of 0.85% sodium chloride (isotonic saline) adjusted to pH 7.4 with 0.1 *N* sodium hydroxide. To this extract was added 50 μL of ^{14}C-labeled anti-human antiserum. This mixture was allowed to remain at room temperature for 2 h. Then 1.0 mL of 0.03 *M* zinc sulfate ($ZnSO_4$) was added to the solution, and the mixture was vortexed and incubated at room temperature for an additional 20 min. Zinc sulfate precipitates any hemoglobin present in the extract by formation of an insoluble complex of the heme fraction of the hemoglobin molecule and the zinc [*8*]. The mixture was then centrifuged for 20 min at 500 $\times g$, and the supernatant was removed and retained for counting. The precipitate was washed with additional zinc sulfate solution and recentrifuged for 20 min. The precipitate was dissolved in 1.0 mL of 1.0 *M* EDTA disodium salt. EDTA chelates the zinc, allowing the hemoglobin to be solubilized. The solution was transferred to a scintillation vial, mixed with 5.0 mL of scintillation fluid (Packard Instrument Company, Downers Grove, Illinois) and counted. The RIA was always run with at least two substrate blanks and two known human stains to serve as negative and positive controls. All samples were run in triplicate, adjusted for background counts (ranging from 15 to 30 CPM), and mean CPM scores calculated and recorded. Specific activity denotes the CPM scores produced per 50 μL of blood (7.5 mg of hemoglobin).

Determination of Human Specificity and Sensitivity—The RIA was applied to the analysis of fresh 50-μL bloodstains produced from human, horse, cow, rat, dog, cat, sheep, goat, rabbit, chicken, rhesus monkey, baboon, or chimpanzee bloods. Bloodstains were always produced on cotton fabric substrate unless otherwise stated. Test specimens with CPM greater than three standard deviations above the mean value obtained with bloodstains from the species with results closest to those obtained from human bloodstains were considered positive.

The sensitivity of the technique was determined by applying the RIA to 50-μL aliquots from each of ten serially diluted solutions of human whole blood. The level of sensitivity is defined as the amount of human hemoglobin needed to give a positive result for human blood.

Effect of High Levels of Variant Human Hemoglobin in Bloodstains on RIA—Because of the possible antigenic differences between variant human hemoglobins and normal human hemoglobin (hemoglobin A_1), the anti-human hemoglobin antibodies may not recognize and bind to human hemoglobins other than A_1. If this is true, then human bloodstains with high levels of variant hemoglobins may give a weak or negative result with the RIA. To check this possibility, the RIA was run on saline extracts of fresh 50-μL human bloodstains prepared on a cotton fabric substrate and containing high levels of hemoglobin S and hemoglobin F, respectively.

Effect of the Age of Human Bloodstains on RIA—The RIA was applied to extracts of human bloodstains of varying ages up to six years old to determine the effect, if any, of the age of the bloodstain on the RIA. Each bloodstain was produced on a cotton fabric substrate

and maintained desiccated at room temperature. Any observed effect is presumably related to structural changes of the hemoglobin molecule in the bloodstain over time.

Three specimens, approximately 1 by 1 cm, were cut from each bloodstained fabric, extracted, and assayed using the RIA technique.

Effectiveness of RIA Using Human Bloodstains Exposed to Various Environmental Influences—Experiments were conducted to determine if dried human hemoglobin can be altered to such a degree by environmental conditions that its human origin cannot be demonstrated by the radioimmunoassay.

1. Effect of Temperature: Fresh 50-μL human bloodstains prepared in triplicate on cotton fabric substrate were incubated at −16, 7, 24, or 37°C for six weeks. After this time, the RIA was applied to each of the bloodstains. The effect of temperature on dried human hemoglobin can then be noted by observing any deviation of the results obtained from bloodstains incubated at −16, 7, or 37°C from those obtained at 24°C (room temperature).
2. Effect of Humidity: Fresh 50-μL human bloodstains on cotton fabric were incubated in triplicate in nonaseptic chambers having 0, 33, 67, or 98% relative humidity for three weeks. After the three-week incubation period, the RIA was run on the bloodstains. The effect of humidity on dried human blood can then be determined by observing any deviation of the results obtained from bloodstains incubated at 0, 33, 67, or 98% relative humidity from the results obtained from bloodstains incubated under dry conditions.

Since humidity promotes bacterial growth, the observation of a significant effect may be due to bacterial contamination of the stain and not directly due to humidity. A study, therefore, was conducted to determine the effectiveness of the RIA on bacterially contaminated human bloodstains.

3. Effect of Bacterial Contamination: Fresh 50-μL human bloodstains (cotton fabric substrate) were inoculated with either gram-positive bacteria (*Bacillus subtilis*) or gram-negative bacteria (*Escherichia coli*) and placed in a moisture chamber for two weeks. Three specimens were inoculated in each case. After the two-week incubation period, the RIA was performed on the bloodstains. The effect of bacterial contamination was then observed by comparing the results obtained from the contaminated bloodstains with those from noncontaminated controls.

Results

To determine if the rabbit anti-human hemoglobin antiserum exhibits cross-reactivity to nonhuman primate blood components, the Ouchterlony double immunodiffusion method was used. Single-precipitin bands developed between the wells containing the anti-human hemoglobin antiserum and saline extracts from human, baboon, rhesus monkey, and chimpanzee bloodstains, respectively. Thus, cross-reactivity is exhibited with hemoglobin from these particular species. Although the position of the precipitin bands was identical, the intensity of the bands between the wells containing the antiserum and the human bloodstain extract was greater than those of the other three species despite the fact that equal volumes of extract were allowed to diffuse, indicating that antigen-antibody complex formation was greatest with human blood. The precipitin pattern did not exhibit spurs or hooks. The undiluted antiserum was not reactive with bloodstain extracts from any nonprimate species tested.

Figure 1 shows that human bloodstains are easily distinguished from bloodstains originating from all other species tested by RIA. Results obtained with human bloodstains reveal about a fivefold increase in CPM over bloodstains from nonhuman primates (baboon, chimpanzee, rhesus monkey), while showing about a 50-fold increase in CPM over other species.

In this study, the minimum value accepted as a positive result is 1700 CPM. This value is approximately three standard deviations above the mean value obtained with three blood-

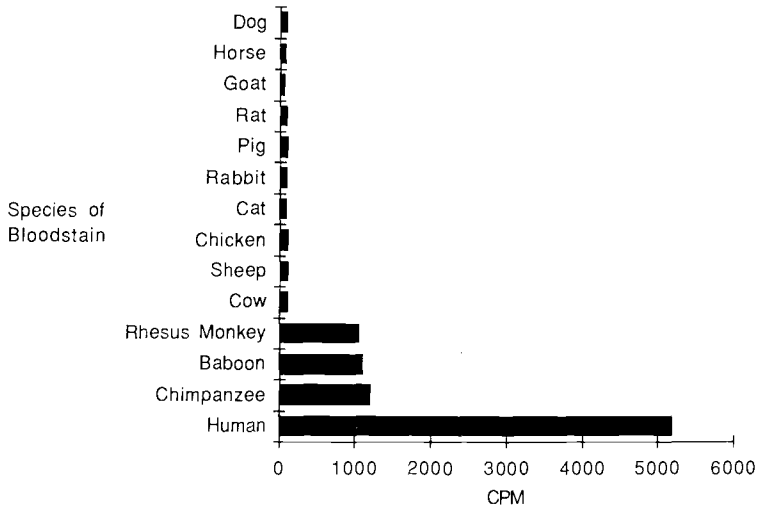

FIG. 1—*Species specificity of RIA: 50-μL blood specimens from various species were used to produce bloodstains on cotton fabric substrates. After a prescribed period of time, the stains were extracted and treated as described in the Materials and Methods section.*

stains, tested in triplicate, from the species producing results closest to those of human (chimpanzee).

Figure 2 shows the results obtained with serially diluted solutions of 50 μL of human whole blood using RIA. The maximum dilution of 50 μL of human whole blood able to give a positive result was determined to be 1:32 (approximately 0.8-μL human whole blood and 0.12-mg hemoglobin). All specimens were run in triplicate and mean values recorded.

Positive results were obtained with 50-μL bloodstains prepared from newborn human

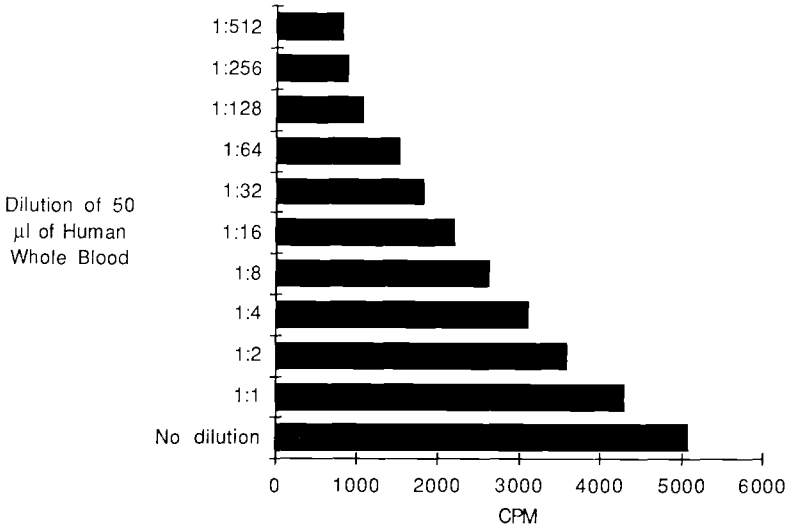

FIG. 2—*Sensitivity of RIA: 50 μL of human whole blood was tested neat or following dilution with isotonic saline.*

blood (19.5% hemoglobin F), sickle-cell blood (hemoglobin S), and normal adult human blood (hemoglobin A_1) (Fig. 3). Analysis of bloodstains prepared from newborn infants produced results indistinguishable from those prepared with normal adult human bloodstains, while those produced with sickle-cell bloodstains yielded only a 16% decrease.

Positive results were obtained with extracts of aged human bloodstains ranging from one month to six years of age which had been maintained desiccated at room temperature (Fig. 4). One-month-old bloodstains yielded results 16% lower than fresh controls. Very little deviation was noted in bloodstains ranging from two months to two years in age. The two-year-old bloodstain extracts showed, on average, a 39% decrease in CPM compared to fresh controls. A 65% decrease was observed with the six-year-old stains.

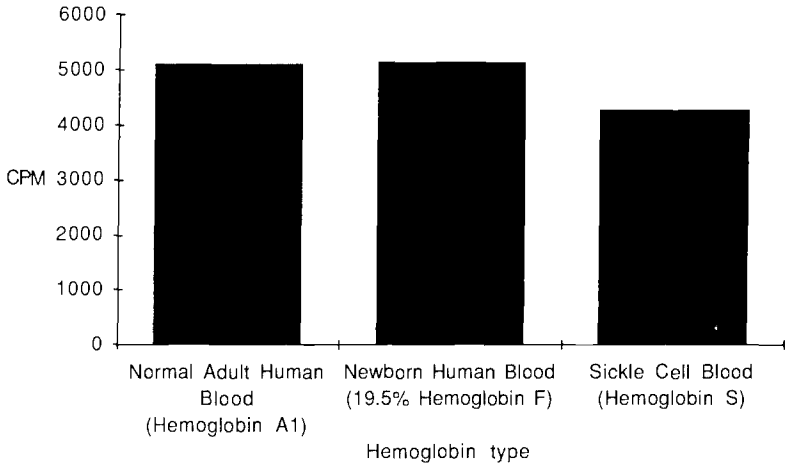

FIG. 3—*Effect of the presence of relatively high levels of variant human hemoglobin in bloodstains on RIA.*

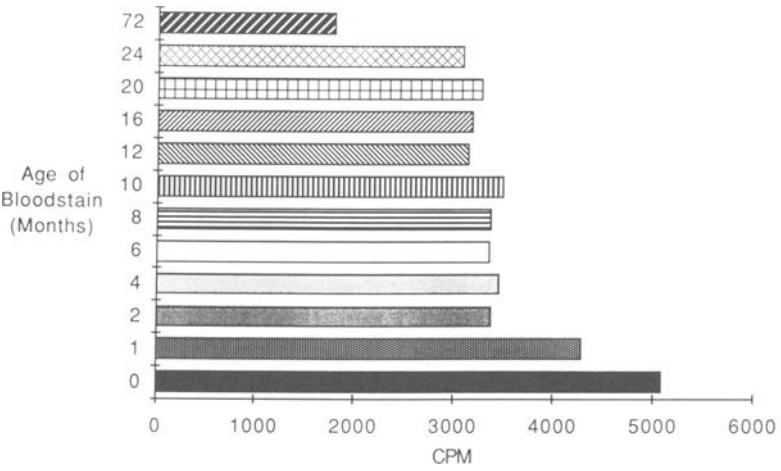

FIG. 4—*Effect of age of the human bloodstain on RIA. Specimens were maintained desiccated throughout the aging process.*

Positive results were obtained with extracts of 50-μL human bloodstains stored at −16, 7, 24, or 37°C for six weeks (Fig. 5). Dried human bloodstains stored at 37°C produced only a 12% decrease in CPM compared to those obtained from bloodstains stored at −16, 7 or 24°C.

Positive results were obtained with extracts of 50-μL human bloodstains exposed to 0, 33, 67, or 98% relative humidity under nonsterile conditions for three weeks (Fig. 6). Fewer counts per minute were obtained with the RIA on extracts of bloodstains exposed to higher levels of relative humidity. Extracts of bloodstains exposed to 33% relative humidity exhibited an 11% decrease in CPM compared with bloodstains which had been maintained dry,

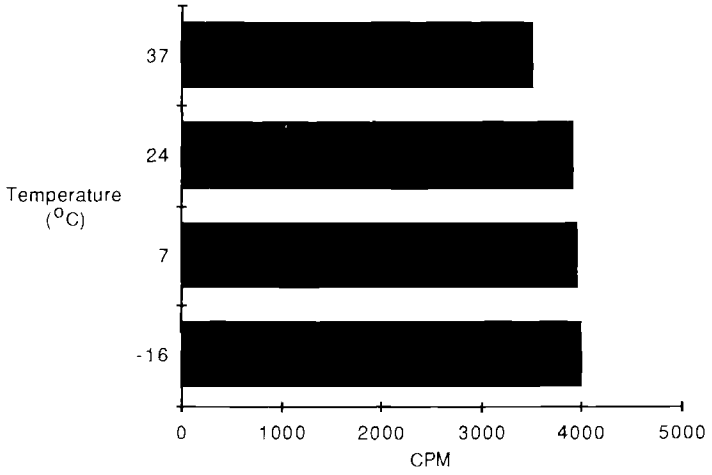

FIG. 5—*Effect of storage temperature on 50-μL human bloodstains stored for six weeks on hemoglobin detection by RIA.*

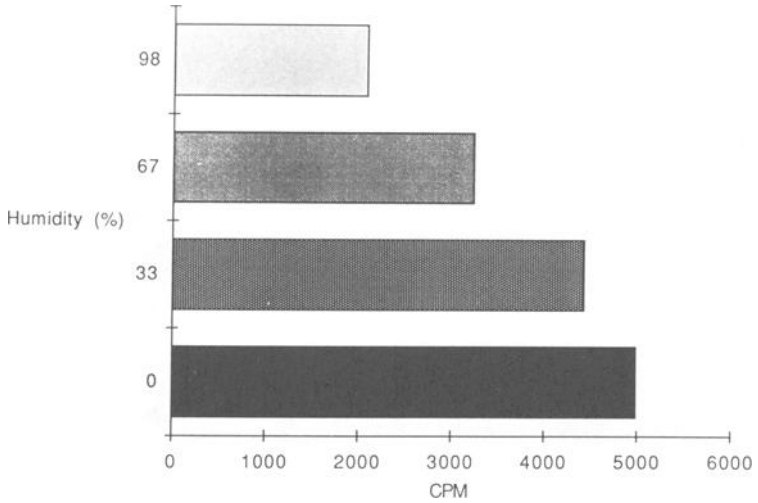

FIG. 6—*Effect of relative humidity on 50-μL human bloodstains stored for three weeks on hemoglobin detection by RIA.*

while those exposed to 67% relative humidity showed a 35% decrease in CPM. A 58% decrease in CPM was noted when bloodstains were maintained at 98% relative humidity.

Positive results were obtained with extracts of 50-μL human bloodstains exposed to bacterial contamination (gram positive or negative) under humid conditions for two weeks (Fig. 7). Bloodstains inoculated with gram-negative bacteria (*Escherichia coli*) showed a 31% decrease from bloodstain controls, while bloodstains inoculated with gram-positive bacteria (*Bacillus subtilis*) showed a 51% decrease.

Discussion

That there is some cross-reactivity of the antiserum with nonhuman primate blood is not surprising since the amino acid sequence of the globin chains of hemoglobin from such species as chimpanzee is almost identical to that of human [*9*]. The genes that control the synthesis of the globin components in human hemoglobin have both unique as well as evolutionarily conserved sequences. Thus, phylogenetically closely related hemoglobins share certain antigenic features, while at the same time remaining unique molecules. Polyclonal antibodies that recognize antigenic determinants present on two different species of hemoglobins will combine with both, while those which are specific for unique sequences will bind differentially. The experimental results reflect this. We have found that compared to extracts of human bloodstains, those of nonhuman primates (baboon, chimpanzee, rhesus monkey) exhibit roughly 20% of the specific activity (CPM). Extracts of bloodstains derived from other species studied exhibit approximately 2% of the activity observed with human bloodstain extracts.

Human bloodstains with high levels of variant human hemoglobins, such as hemoglobin S and hemoglobin F, show no significant differences from normal adult human hemoglobin (A_1) in results obtained with the RIA. It would appear that the purified, radiolabeled antibodies can be successfully used in an RIA to distinguish human from nonhuman hemoglobin, but not to distinguish between different common variants of human hemoglobin. Presumably, the tertiary and quaternary structure of the hemoglobin F and hemoglobin S

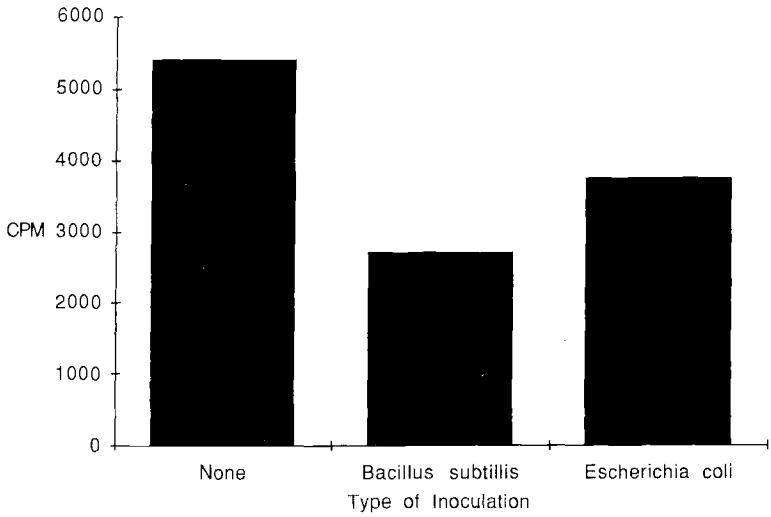

FIG. 7—*Effect of bacterial contamination of 50-μL human bloodstains. Specimens were stored for two weeks under humid conditions and then analyzed by RIA.*

molecules do not differ sufficiently from that of the hemoglobin A_1 molecule to differentiate them using this serological probe.

Bloodstain aging does appear to alter the binding of the antibodies to the hemoglobin molecule, but the effects of the process do not compromise the assay since positive results were obtained with extracts of human bloodstains up to six years old. The effect of bloodstain aging can be seen by the fact that human bloodstains up to two years old exhibited a 39% decrease in specific activity compared to fresh controls. This decrease may be due to a loss of the structural integrity of the hemoglobin molecule in regions where binding to antibodies occurs, to a masking of the binding site or sites, to a loss in hemoglobin content of the dried stain over time, to incomplete extraction of hemoglobin, especially from older stains, or to a combination of some or all of the above.

Environmental factors such as temperature and bacterial contamination did not significantly alter the serological reactivity of the hemoglobin molecule within the dried bloodstain, since positive results were obtained with all bloodstains tested. Indeed, in the dry state, the human hemoglobin molecule may be more resistant to these environmental influences than it would be in its native wet state. In any case, we have observed that environmental factors, such as humidity, temperature, and contamination can and do affect the results of the RIA since specific counts per minute decrease when bloodstains are exposed to these conditions.

In this study, a minimal volume of 0.8 μL of fresh human blood (containing approximately 0.12-mg hemoglobin) was needed to produce a bloodstain sufficient to yield a positive result. Sensitivity levels have been reported to be significantly lower for precipitin methods [*1*]. In general, RIA should be able to detect antigens in the nanogram to microgram range, while precipitin tests are useful when antigens are present in the microgram to milligram range. The RIA described in this report is capable of detecting hemoglobin levels lower than 0.12 mg; however, the sensitivity of the assay is dependent on the definition of a positive result. This minimum value was set at 1700 CPM, a number more than three standard deviations above the mean value obtained with chimpanzee bloodstains as described in the foregoing. While this value minimizes the possibility of obtaining false positive results, it still provides for an assay sensitivity that is far greater than that obtained with precipitin tests. Furthermore, the RIA technique for the identification of human hemoglobin described in this paper is also more sensitive than the RIA procedure described by Butt [*4*]. The latter assay is used to determine the human origin of a bloodstain by detecting the presence of human beta-thromboglobulin (BTG). Approximately 2 μL of human whole blood are required to produce positive results using this method.

The RIA for BTG suffers from two disadvantages. First, the availability of commercial antisera containing antibodies specific for human BTG is limited. Second, the stability of BTG in dried human bloodstains over time and under various environmental conditions has not been adequately studied.

The criterion for a positive result was established to be a background-corrected CPM value of at least three standard deviations above the mean value obtained with three fresh 50-μL bloodstains from the species producing results closest to those obtained from human stains, that is, chimpanzee. In this case, the minimum value for a positive result was set at 1700 CPM since chimpanzee produced a mean value of 1217 ± 140 CPM. This number was chosen as the cutoff level since even under maximum response conditions, chimpanzee bloodstains can be easily differentiated as nonhuman.

The RIA described here has been developed using known quantities of blood and well-characterized reagents. Its usefulness in the forensic field must still be demonstrated, however. Note that the assay described in this report will not produce positive results with human serum stains since the technique depends upon the binding of antibody with hemoglobin. Remember that the sample volume of bloodstain is usually not known in most forensic science situations. Thus, a bloodstain consisting of a large amount of nonhuman primate blood could produce a positive result using the criterion described above. However, the probability

of finding such a bloodstain is extremely low in most parts of the world. It is also doubtful that bloodstains containing large volumes of bloods from common animals, such as dog or cat, will produce significant specific counts per minute with the RIA since very low results were obtained in this study with 50 μL bloodstains. A way to minimize the problem of unknown bloodstain volume might be to determine spectrophotometrically heme absorption of the bloodstain and estimate the total amount of hemoglobin present. This is an important consideration that merits further study.

Acknowledgments

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References

[1] Lee, H. D., "Identification and Grouping of Bloodstains," *Forensic Science Handbook*, Richard Saferstein, Ed., Prentice-Hall, Englewood Cliffs, NJ, 1982, pp. 267-337.

[2] De Forest, P. R. and Lee, H. C., "Routine Use of Anti-Human Hemoglobin Serum in Bloodstain Identification," paper presented at the 29th Annual Meeting of the American Academy of Forensic Sciences, San Diego, CA, 1977.

[3] Garver, F. A. and Talmage, D. W., "The Identification of the Primary Interaction Between ^{125}I-Labeled Human Hemoglobin and Rabbit Anti-Human Hemoglobin Sera: A Sensitive Radioimmunoassay Technique," *Journal of Immunological Methods*, Vol. 7, 1975, pp. 271-282.

[4] Butt, R. W., "Identification of Human Bloodstains by Radioimmunoassay," *Journal of the Forensic Science Society*, Vol. 23, 1983, pp. 291-296.

[5] Ouchterlony, O., "Antigen-Antibody Reactions in Gels," *Acta Pathologica et Microbiologica Scandinavica*, Vol. 26, 1949, pp. 507-515.

[6] Means, G. E. and Feeney, R. E., "Reductive Alkylation of Amino Groups in Proteins," *Biochemistry*, Vol. 7, 1968, pp. 2192-2201.

[7] Feeney, R. E. and Osuga, D. T., "Purification of Chemically Modified Proteins," in *Methods of Protein Separation*, N. Catsimpoolas, Ed., Plenum Press, New York, 1975, pp. 127-159.

[8] Valentine, W. N. and Paglia, D. E., "Partial Purification of Some Red Cell Enzymes Utilizing Hemoglobin Precipitation by Zinc Salts," *Journal of Laboratory Clinical Medicine*, Vol. 101, 1983, pp. 617-622.

[9] Lehman, H. and Huntsman, R. G., *Man's Haemoglobins*, North Holland Publishing Company, Amsterdam, 1966, pp. 89-90.

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