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The Detection of Y Chromosomes in Bloodstains—A Reevaluation

Traditional approaches to the grouping of bloodstains in forensic science involve a comparison of blood groups found in a stain with those of the suspect or victim involved in a crime. However, in some instances, such as those crimes in which no control blood samples are available, the blood grouping data alone are of little value to either the police officer or the courts. Information, however, relating to an individual's physical appearance, such as sex or age, which can be derived from a bloodstain is of value even in the absence of control blood. Such an approach to bloodstain investigation has been discussed earlier [*1*] and forms the philosophic basis of the present work concerned with the determination of the sex of the donor of a bloodstain.

The visualization of chromosomes in the metaphase nucleus by use of the fluorochrome stain quinacrine mustard was first reported by Caspersson et al [*2*]. Subsequently, Zech [*3*] reported that this stain caused the human Y chromosome to fluoresce particularly brightly. These findings were confirmed by Pearson et al [*4*], who also showed that the Y chromosome was detectable during interphase by using the closely related stain quinacrine dihydrochloride. This report was followed by the adaptation of the technique for use in various biological fields; for instance, Schwinger [*5*] reports detecting the Y chromosome (or two Y chromosomes in XYY individuals) in various tissues, namely the lymphocytes of blood, the sheath cells of hair roots, and the heads of spermatozoa.

In the field of forensic science, Phillips and Gaten [*6*] developed a method for determining the sex of the donor of a bloodstain. This was followed by a comprehensive study on the sex identification of human blood and bloodstains by Ishizu [*7*] and by Lyubinskaya and Antonova [*8*], who published their findings on the incidence of the Y chromosome in blood smears and bloodstains. More recently, Kringsholm et al [*9*] described a staining method for the sexual discrimination of hairs and bloodstains.

The "Y spot" is normally seen as a bright chromocenter in a quinacrine dye-stained nucleus. The intensity and size of the spot vary, presumably reflecting the variable size of the Y chromosome in man [*10,11*], and the degree of background stain of the nucleus considerably affects the ease with which the mass can be distinguished. It may appear as a spot or as a crescent, either at the edges or in the center of the nucleus. The technique

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is therefore very subjective, and Phillips and Gitsham [*12*] consider that "each person's judgement needs to be 'calibrated.' " Also, some form of fluorescent material occasionally appears in female cells that may be confused with "true" Y spots from male cells and this may make interpretation difficult. Kegel and Conen [*13*] consider this fluorescence from female nuclei as resulting from "either a non-specific condensation of chromatin or strongly fluorescent portions of other chromosomes."

The present study is aimed at further understanding the factors that influence the Y spot count found in leukocytes from bloodstain extracts. The subjectivity of the method has been assessed, the effect of type of substrate and age of stain has been considered, and finally a "blind" trial involving bloodstains derived from case material is reported.

Materials

Quinacrine dihydrochloride was obtained from Sigma Chemical Co. All other reagents used were of Analar® grade and were obtained from BDH Chemicals Ltd.

Blood smears and bloodstains were made from blood obtained by fingerprick. Several substrates were used for the bloodstains: cotton, glass, nylon, paper tissue, polyester, triacetate, acrylic, and aluminum. The bloodstains ranged from 1 to 38 days old at the time of examination.

Prepared bloodstains were examined with either a Reichert Zetopan microscope, fitted with an HBO 200-W mercury vapor lamp, BG 38 and FITC3 exciter filters, dark ground condenser, and a GG9 barrier filter, or a Leitz Orthoplan microscope fitted with a Ploemopak 2.1 epi-illuminator employing the H filter cube combination. Both microscopes were used at an optical magnification of ×500.

Methods

Extraction and Staining Techniques

Blood Smears—Thin blood smears were made on 76- by 25-mm glass microscope slides. They were allowed to dry at room temperature before fixation in 100% methanol for 15 min. The fixed smears were then stained in 1% aqueous quinacrine dihydrochloride for 15 min, washed thoroughly in distilled water, and mounted in phosphate buffer, pH 5.4 [*14*].

Bloodstains on Cloth—A small bloodstain, approximately 0.5 by 0.5 cm, was cut into pieces of about 5 by 1 mm and placed in a centrifuge tube. Then 0.5 ml of 2 m*M* magnesium chloride, after the method of Phillips and Webster [*15*], was added before grinding was done with a blunt glass rod to extract the cellular material. This grinding procedure was repeated every 15 min for 2 h.

The maximum amount of liquid was removed from the cloth by pipet and placed in a fresh centrifuge tube and spun for 1 min at 7000 *g*. The supernatant obtained was discarded and the pellet resuspended in about 0.5 ml of fresh 2 m*M* magnesium chloride. The suspension was recentrifuged as previously described and the supernatant again discarded. The washed pellet was mixed with approximately 100 μl of 2 m*M* magnesium chloride, and then the fixative, 3:1 v/v methanol/glacial acetic acid, which must be prepared immediately before use, was added by drops up to a maximum volume of 0.75 ml.

The tube was incubated at room temperature for 10 min and occasionally shaken. It was then centrifuged as previously described and the resulting pellet was transferred, by Pasteur pipet, in as little volume of fixative as possible to a methanol-cleaned microscope slide. The pellet was smeared on the slide and allowed to dry at room temperature before incubation at 56°C for 15 min to completely dry the slide.

After cooling, the slide was stained as described for blood smears.

Bloodstains on Solid Substrates—One drop of 2 m*M* magnesium chloride was added directly to the bloodstain and a glass rod was used to stir in the extractant until the bloodstain was taken up in suspension. The suspension was then decanted into a centrifuge tube and incubated at room temperature for 2 h with occasional shaking. The procedure for bloodstains on cloth was then followed from the first centrifugation.

Microscopy

Slides were examined within 1½ h of preparation. This avoided loss of definition of Y chromosomes resulting from fluorescent decay. A minimum of 50 intact leukocyte nuclei (excluding the polymorphs) was examined on each slide. The percentage of positive nuclei, that is, those with a visible Y chromosome, was calculated and termed the Y cell index.

Procedure

Blood Smears—Blood smears from 27 males and 10 females were examined by open trial, that is, the sex of the donor of the sample was known to the operator. The results obtained have been compared with a similar trial conducted blind, that is, the operator did not know the composition of the trial at the time of examination. This involved blood smears from 23 males and 18 females.

Bloodstains—An open trial involving bloodstains made on cotton cloth from 36 males and 12 females was carried out. The age of these stains at the time of examination ranged from 1 to 38 days old. Approximately twice as many similar bloodstains were examined by blind trial.

Also, a blind trial was conducted with bloodstains on various substrates (see Materials section). The stains were donated by 21 males and 23 females and at the time of examination the stains were between 4 and 22 days old.

To determine the effect of substrate on the Y cell index, bloodstains were made by six male donors on three contrasting substrate types. These were a nonabsorbent substrate, glass; a synthetic cloth, nylon; and a natural cloth, cotton. They were examined by blind trial between four and seven days after preparation.

Blind Trial Involving Case Material—The eight operational forensic science laboratories in England and Wales were requested to submit, blind, bloodstained material from cases in which from other evidence there was no doubt as to the sex of the donor (for example, the underclothing of the victim of a stabbing attack where there was no doubt of the origin of the blood).

One hundred thirty-nine bloodstains were submitted to our laboratory for analysis. In this instance the bloodstains were reported as being of male origin if the Y cell index was not less than 25%; other stains were reported as inconclusive. Results were reported in this manner to the laboratory submitting the stains who subsequently informed us of the correct result.

Results and Discussion

The optimal viewing conditions were found to be a very dark background in which the nuclei fluoresced a bright green. The Y chromosome was seen as a small discrete white fluorescent spot on the nucleus. Generally the Y spot was located peripherally (Fig. 1), but occasionally it was found towards the center of the nucleus.

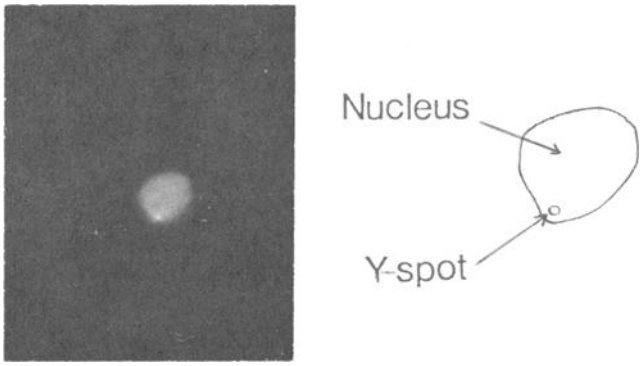

FIG. 1—*Photograph and diagram of a Y chromosome in a leukocyte nucleus.*

Blood Smears

In both our trials, no females had a Y cell index greater than 10%, while male scores ranged from 20 to 60% with one exception. Male Y cell indexes were generally higher when the sex of the donor was known: the mean was 48.4% in the open trial (Fig. 2) compared with 38.6% for the blind trial (Fig. 3). This is reflected by the difference between the lower range value for the males and upper range value for the females, that is, the separation of the male/female population: 18 percentage points for the open trial compared with 10 percentage points for the blind trial. These blood smear results confirm that there is a difference between the Y cell indexes of males and females reported by other workers (Table 1).

Thus from our results and from the literature for fresh blood smears a Y cell index of >20% indicates a male donor and a Y cell index of <10% probably indicates a female donor.

Bloodstains on Cotton Cloth

Bloodstains on cotton cloth examined in the open trial essentially gave results parallel to those obtained from our blood smear trials: male mean index, 42.4% and female mean index, 0.8% (Fig. 4). However, the results obtained from the blind trial of bloodstains on cotton cloth (Fig. 5) were less discriminating between males and females than in the

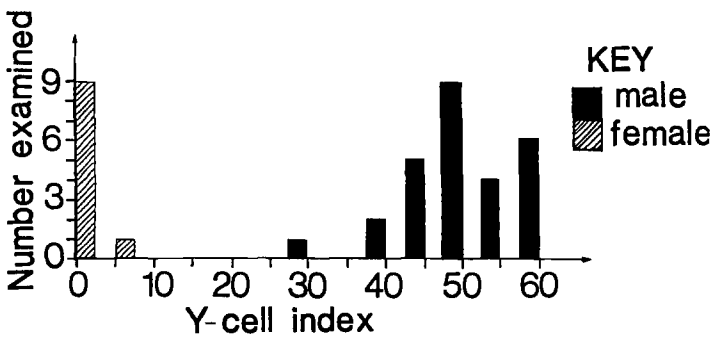

FIG. 2—*Histogram plot of Y chromosome fluorescence in fresh blood smears, open trial results. Male mean index, 48.4%; range 26 to 60%. Female mean index, 1.8; range, 0 to 8%.*

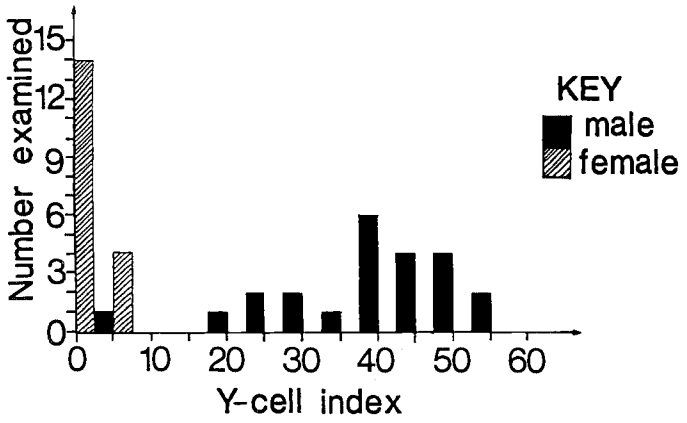

FIG. 3—*Histogram plot of Y chromosome fluorescence in fresh blood smears, blind trial results. Male mean index (excluding one result), 38.6; range, 20 to 54%. Female mean index, 2.7; range, 0 to 10%.*

open trial. Approximately 30% of the males scored less than 20%. These results together with those from the blind trial on blood smears emphasize the subjective nature of this technique.

Bloodstains on Varied Substrates

Better discrimination of males/females was achieved in the varied substrates blind trial (Fig. 6) than in the cotton cloth blind trial. Females scored a Y cell index of 16% or less (mean 3.4%) and males 14% or more (range, 14 to 64%; mean, 35.1%). This apparent discrepancy may be resolved by reference to Fig. 7, which reveals that cotton is a poor substrate; cotton gave statistically poorer results than both nylon (significant at the 5% level) and glass (significant at the <5% level), which gave essentially similar results (that is, glass is not significantly different from nylon). Thus the results obtained

TABLE 1—*Y cell indexes of leukocyte nuclei reported by us and other workers.*

Author	Y Cell Index, %	
	Male Blood Smears	Female Blood Smears
Ishizu [7] by open trial	47-88 avg = 62.6	0-4 avg = 0.5
Lyubinskaya and Antonova [8] by open trial	46-90 avg = 74	0-3 avg = 1.1
Conen et al [16] by open trial	80.1	4.5
Polani and Mutton [17] by open trial	61-87 avg = 69	0-13 avg = 5
Phillips and Gaten [6] by blind trial	86 ± 12.3	0.5
Kringsholm et al [9] by blind trial	lowest (with 1 exception) = 32	highest = 4
This paper		
Open trial	26-60 mean = 48.4	0-8 mean = 1.8
Blind trial	20-54 mean = 38.3	0-10 mean = 2.7

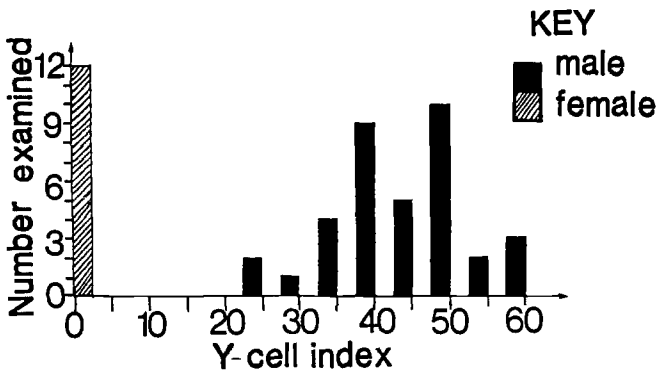

FIG. 4—*Histogram plot of Y chromosome fluorescence on cotton cloth, open trial results. Male mean index, 42.4%; range, 22 to 60%. Female mean index, 0.83%; range, 0 to 2%.*

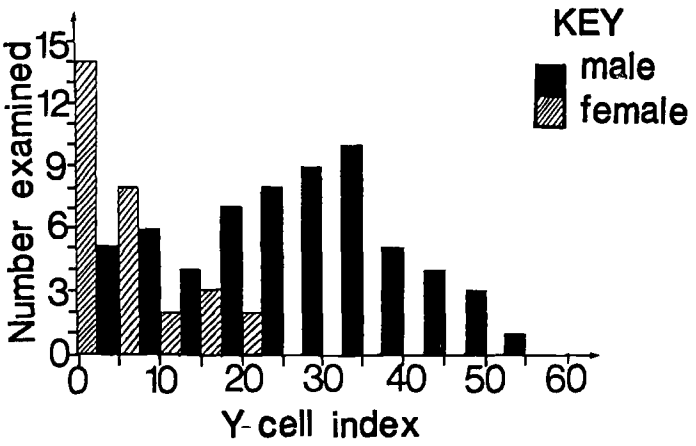

FIG. 5—*Histogram plot of Y chromosome fluorescence on cotton cloth, blind trial results. Male mean index, 25.1%; range, 0 to 54%. Female mean index, 7.4%; range, 0 to 24%.*

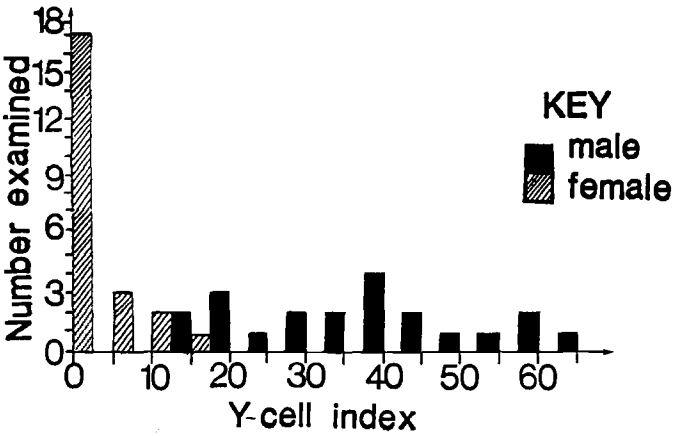

FIG. 6—*Histogram plot of Y chromosome fluorescence from bloodstains on various substrates, blind trial results. Male mean index, 35.1%; range, 14 to 64%. Female mean index, 3.4%; range, 0 to 16%.*

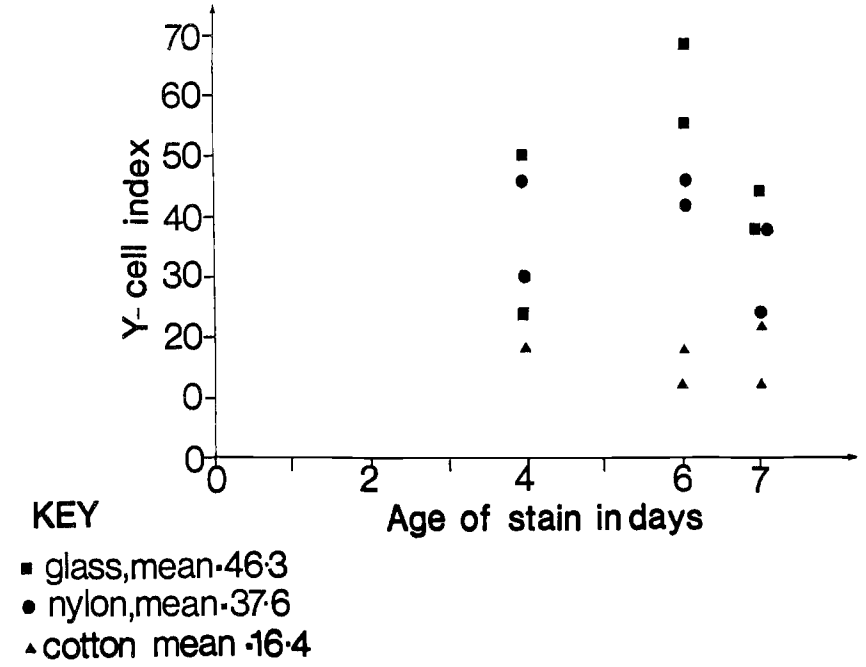

FIG. 7—*Effect of substrate on Y chromosome fluorescence in bloodstains from male donors, blind trial results. Mean value for glass, 46.3%; mean value for nylon, 37.6%; and mean value for cotton, 16.4%. (Cotton Stain 2 on Day 4 was not readable.)*

from the bloodstains made on cotton were the most difficult to interpret and therefore probably represent the most problematical situation. Earlier workers have suggested that cotton "destroys" the leukocytes [*18*], but it appears probable that difficulty of extraction is the reason for the lower Y cell indexes.

Bloodstains made on certain natural substrates such as leather, coarse wool, and wood were difficult to analyze for Y chromosomes. In the case of wood the debris unavoidably obtained during the extraction procedure was highly fluorescent, making it impossible to identify the leukocyte nuclei. Wool and leather extracts were characterized by an abundance of highly fluorescent globules, which similarly obscured the leukocyte nuclei.

Brinkman and Jobst [*19*] investigated, by blind trial, bloodstains up to four weeks old made on a variety of substrates and reported male scores of 12.5 to 50% and female scores of 0 to 7.9%. They also investigated older stains and were able to observe positive Y cells in stains of male origin up to 28 months old, but beyond this time the Y cell index fell to zero. Ishizu [*7*] also investigated bloodstains from three days to six months old on six or seven different substrates and reported male scores of 17 to 67.5% and female scores of 0 to 3%. In more recent blind trial studies, Kringsholm et al [*9*] examined bloodstains on cotton cloth up to four weeks old. They found in those of male origin a Y cell index range of 26 to 70%. Bloodstains of female origin had a range of 0 to 4%.

In addition, Kringsholm et al [*9*] found that the Y cell index of male bloodstains fell with time, after nine weeks to 20 to 24%. Similarly, Phillips and Gaten [*6*], who examined portions of a large bloodstain on glass from a male donor, found at Day 0 that a Y cell index of 30% was obtained but that this value decreased to 3% after ten days; subsequently, with an improved extraction method, Phillips and Webster [*15*], using bloodstains on stainless steel, reported scores of about 45% for male donors at Day 0, decreasing to 8.5% and 7% after four and eight weeks, respectively. Similar experiments with

linoleum gave 70% at Day 0, 15% at four weeks, and 10% after eight weeks. In contrast, our results on case bloodstains up to 138 days old (Fig. 8) do not show any decrease with the age of stain.

Blind Trial on Case Material

The results of the case-work analysis appear encouraging. Of the 139 bloodstains examined, 89 were from male donors. We correctly identified 57 of these (that is, 65% of the male stains were detected). The other 32 male stains were classified as inconclusive. The mean Y cell index for males was 31.5%, and the range was 0 to 68%. Also, of the 139 bloodstains, 50 were from females, none of which was incorrectly reported as of male origin. The mean Y cell index was 4.3% and the range, 0 to 14%. Where a Y cell index of <25% was obtained, the stain was reported as inconclusive. This group may include stains of female origin and male stains from which white blood cells with Y chromosomes for whatever reason are unextractable from the substrate, and chromosomal abnormalities [*20*] may account for a very small proportion of males that are undetectable by this technique.

Once the full results relating to each stain were available it was possible to analyze the data further, for example, correlating the Y cell index with the age of the stain as given in Fig. 8. It can be seen that a cutoff point of >25% Y cell index is well above the mean female index of 4%, the upper level being 14%. It is also clear that again there is no obvious reduction of the Y cell index with time, the majority of the bloodstains being in the range 0 to 80 days old, and a few extending up to 138 days old, at the time of examination.

Microscopy

In general, the sexing of bloodstains proved more difficult than blood smears.

To obtain meaningful results it is essential that at least 50 nuclei be examined on every slide. This procedure was followed rigorously in all cases. In contrast, Kringsholm et al [*9*], while recommending that a similar procedure should be followed, in practice found that bloodstains on cotton cloth were particularly difficult to extract. Consequently

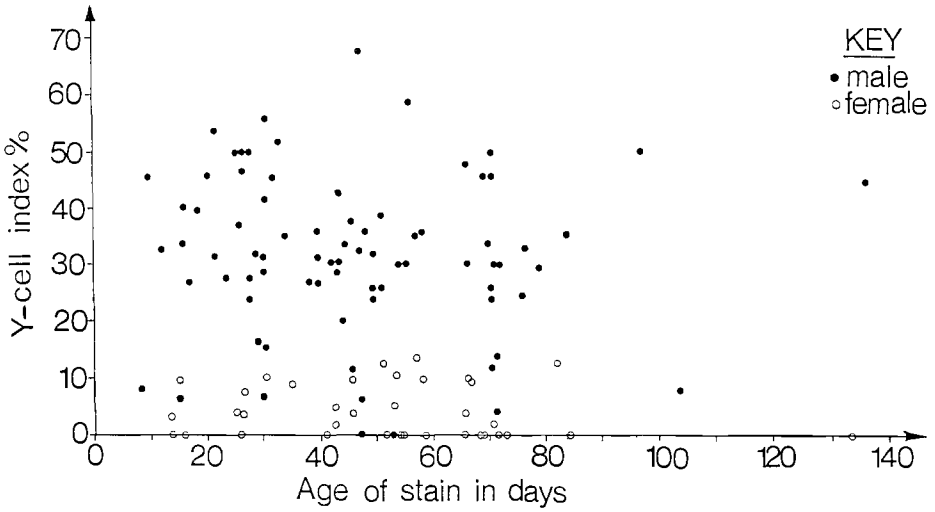

FIG. 8—*Results for bloodstains from case work, examined by blind trial.*

50 nuclei were examined in less than 50% of their cases. Our experience has been that to obtain the maximum number of nuclei from bloodstained cloth it is essential that the extract is ground vigorously at the extracting stage.

A further requirement of this very sensitive technique is that precise optical conditions be used. In our hands the optical arrangement of the Leitz microscope was found to give a superior image to that of the Reichert. Although both instruments proved to be adequate for sexing blood smears, the difference in the image was particularly marked when sexing in the more demanding case of bloodstains.

Conclusion

Our results emphasize that it is essential to employ properly controlled blind procedures to obtain meaningful results from this highly subjective technique. Also, our results illustrate the important effect that substrate has upon the Y cell index. Indeed, this fact alone may explain the apparent discrepancy between published observations. Even variations in the same substrate (for example, cotton cloth) might give rise to inter-laboratory variation.

With our technique it is of some interest that up to an age of stain of 138 days no significant lowering of Y cell index has occurred, which contrasts with previous workers. We have adopted a very cautious approach in reporting male stains on case-work material and use a figure of $\geq 25\%$ as indicative of a male donor. Further experience will dictate whether this can safely be lowered to 20% or less. To ensure accurate reporting in case work, we recommend that the unknown bloodstain be examined in duplicate and also that two control bloodstains be included, a bloodstain from a male with a known high Y cell index and a female control. These four stains should be coded and examined blind, because the interpretation may be influenced a posteriori if the sex of the donor of a bloodstain is known, as in the case of open trials.

Summary

A method has been described for detecting Y chromosomes in the leukocytes of human bloodstains prepared on a variety of substrates. The factors that influence the proportion of chromosomes exhibiting a Y spot (the Y cell index) in a bloodstain are considered, including the subjective nature of assessment of the Y chromosome fluorescence, the substrate, and the age of bloodstain. In contrast to previous workers no decay in Y cell index with the age of the stain was observed.

The results of a blind trial involving stains derived from case work, where from other evidence there was no doubt as to the sex of the donor, are presented. Sixty-five percent of the male bloodstains were correctly identified and no females were wrongly reported as male.

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