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Studies by Crossed Electroimmunodiffusion on the Individuality and Sexual Origin of Bloodstains

The potential value of serologic reactions to forensic studies has been recognized since 1901 when Uhlenhuth [*1*] used the precipitin test to distinguish human from animal blood and Landsteiner [2] used agglutination to show ABO antigenic differences among human red blood cells (RBC). However, the successful use of serologic techniques in criminal cases did not occur until 1916 when Lattes [*3*] devised a simple agglutination test for ABO typing of human bloodstains. The Lattes test, though widely used in the past and improved in various ways [*4–9*], is no longer the method of choice in most forensic laboratories because positive results depend on preservation of the activity of relatively labile antibodies in the stain.

The recognition that, in a dried bloodstain, the RBC antigens are more likely to retain activity than are antibodies led to the development of several tests for detection of such antigens. Most notable among these are absorption elution, first described by Siracusa [*10*] in 1923, and absorption inhibition, developed by Sera [*11*] in 1926. Absorption elution proved to be the more sensitive, and, consequently, with various modifications [*12–16*], it has become the standard technique in most forensic laboratories for ABO typing of bloodstains. Moreover, absorption elution can be used to type bloodstains according to the MN [*17, 18*], Rh [*19, 20*], and Kell [*21*] systems, although with more difficulty than with the ABO system.

The typing of blood stains according to the various antigenic systems on RBC, and to a lesser extent on the basis of genetic differences in serum proteins [22], has been of considerable value in criminalistics and other areas by showing that a stain could or could not have come from a particular individual. However, it is important to note that with these approaches the degree of certainty concerning the origin of a stain is directly related to the extent of typing in different groups. Thus, if it is important to provide strong evidence that a stain came from a particular source, one must perform multiple tests which are expensive and often difficult to interpret. Moreover, such typing, no matter how extensive, never permits conclusions of absolute certainty. It seems clear, therefore, that alternative approaches to bloodstain characterization should be explored.

In our view and that of others [*22–25*], there is considerable potential in combined electrophoretic-immunologic reactions in gel because such techniques, in a single run, can reveal subtle qualitative and quantitative differences among individuals in many serum proteins. Thus, Laudel et al [*23*] used immunoelectrophoresis to show that fresh serum samples from nine individuals could be distinguished if identical run conditions

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were employed, and Whitehead et al [24] found gross differences among individual samples in bloodstains when examined by crossed electroimmunodiffusion (CEID). These investigations, although provocative, were only preliminary and did not consider whether individualization would be possible under conditions of normal and unavoidable variations in technique and individual physiology.

We evaluated CEID as a means for individualization by comparing the nature and extent of variation within and among individuals in bloodstain proteins during a four-month period. This method was chosen because it resolves more antigens than any other technique, and, most important, it detects quantitative as well as qualitative differences [25]. Moreover, the demonstration that CEID can reveal significant female-male differences in certain serum proteins [25] prompted us to evaluate the technique as a means for determining the sexual origin of a bloodstain, an extremely valuable, but at present rarely possible, determination [26].

Materials and Methods

Blood Donors

Five males and five females, all college students, provided blood samples on ten different occasions over a four-month period. The ages of the males ranged from 24 to 29 years and those of the females from 22 to 35 years. All donors were in apparently good health during the course of the study.

Bloodstains

Donors' fingers were punctured, and blood was collected on sections of a cotton bed sheet. The blood was allowed to dry at room temperature, in a closed drawer, for 48 h. At the end of the drying time a 6-mm diameter section was cut from the cloth containing the bloodstain and placed in a 2-ml polystyrene beaker containing 0.05 ml of phosphate buffered saline (PBS; 0.85% NaCl buffered to pH 7.2 with 0.02*M* sodium phosphate). After 5 min the eluted blood was withdrawn, protein concentration was measured by the method of Warburg and Christian [27], and sufficient PBS was added to give a final concentration of 10 mg protein per ml.

Antiserums

Antiserum to whole human serum was prepared in two New Zealand white rabbits. Each rabbit was injected subcutaneously with an emulsion of 0.3 ml of human serum and 0.3 ml of Freund's complete adjuvant on each of three alternate days and was bled six days after the last injection. This regimen was continued until each rabbit had been injected 21 times and bled 7 times. The serums from bleedings 4 through 7 were pooled, dispensed into 7-ml aliquots, and frozen at −20°C (−4°F). Aliquots were thawed on the day they were needed and were not refrozen. Monospecific antiserums were obtained from Behring Diagnostics and maintained at 4°C (39°F).

Crossed Electroimmunodiffusion

The CEID analysis was performed essentially as described earlier [28]. Agarose (Bausch and Lomb) was dissolved in boiling tris-barbital-sodium barbital buffer (High Resolution Buffer, Gelman Instrument Co.) of pH 8.8 and ionic strength 0.03 to give a 1% solution. Five millilitres of the molten agarose was pipeted onto a 25 by 75-mm microscope slide and permitted to solidify in a humid chamber. A 3-mm diameter antigen well was then made 15 mm from one end of the slide. For electrophoresis in the first dimension the appropriate volume (see below) of eluted bloodstain was placed in the antigen well and electrophoresed at 350 V for 60 min. The agarose strip containing

the antigens was carefully removed from the slide and transferred to a 50 by 75-mm slide which had been precoated with 0.1% agarose. The remainder of the 50 by 75-mm slide was then uniformly covered with a molten (50°C or 122°F) agarose-antiserum mixture. After solidification of the agarose-antiserum, electrophoresis in the second dimension was conducted at 150 V for 22 h.

After electrophoresis, the nonprecipitated proteins were removed by washing the slide in 0.9% NaCl for 24 h, followed by washing in distilled water for 24 h. The slides were then dried, stained with amido-black, and photographed at a magnification of × 1.6.

To determine optimal amounts of antigen and antibody for routine use in CEID, a series of preliminary assays was done in which the volumes of eluted bloodstains and antiserums were varied 5 μl to 20 μl and from 0.1 ml to 1.0 ml, respectively. Chosen as optimal were 10 μl of eluted stain and 0.3 ml of antiserum (in 3.45 ml of agarose) because these amounts gave distinct and largely nonoverlapping precipitin peaks and patterns that were reproducible and apparently quite different among individuals. The use of larger amounts of antigen or antiserum resulted in the resolution of larger numbers of antigens, but many of the peaks were superimposed, others had no discernible apex and thus their heights could not be measured, and the overall pattern was more difficult to interpret.

Numbering, Measurement, and Identification of Precipitin Peaks

Precipitin peaks were assigned numbers in approximately the order of occurrence from the anodic to the cathodic end of the CEID slide. The Day 41 slide of Subject A, which had 22 peaks, served as the master pattern for locating and numbering the corresponding peaks in all other slides. Peak heights were measured from photographs to the nearest 0.5 mm and then reduced to scale. A line drawn from the center of the antigen well perpendicular to the direction of the second migration served as the baseline for measurements. Some of the peaks were identified as particular proteins by adding 0.1 ml of monospecific antiserum to the agarose-antiserum mixture or 2.0 μl to the antigen well prior to electrophoresis in the first dimension and noting a reduction in the height of one of the numbered peaks. Those peaks identified were (1), α_1-lipoprotein; (2), prealbumin; (3), albumin; (4), α_1-antitrypsin; (5), Gc globulin; (10), ceruloplasmin; (12), haptoglobin; (14), hemopexin; (15), transferrin; (17), α_2-macroglobulin; and (18), immunoglobulin A.

Results

In Fig. 1 are CEID patterns selected to contrast the differences observed within and among individuals during the investigation. Note that bloodstains obtained on different occasions from the same individual gave very similar patterns (Figs. 1*A* and *B*), whereas stains from other individuals gave grossly different patterns (compare Figs. 1*A* and *B* with *C* and *D*). The differences were due largely to variations in the heights of certain peaks (for example, No. 12) and to a lesser extent to the absence of one or more of the 22 peaks in some individuals (for example, Peak 8 was not detected in Subjects F and G). The ranges, means, and undetected peaks for the ten runs for all subjects are shown in Table 1.

In evaluating CEID as a means for individualizing bloodstains it was recognized that although a single run on stains from two subjects could reveal unequivocal differences (as is apparent from an examination of Fig. 1), such differences might not have been observed had the run been made at some other time. Thus, a more rigid and desirable criterion for individualization would be the demonstration that in multiple determinations the range in one or more peak heights for one individual does not overlap the range of the same peaks for another individual. Table 2, which is divided into

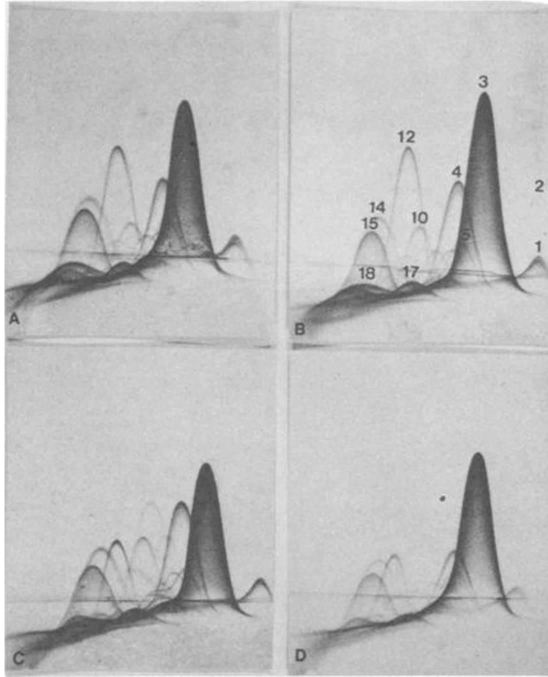

FIG. 1—*Crossed electroimmunodiffusion patterns of human bloodstains.* (A) *Subject A, day 24.* (B) *Subject A, 41. Numbered peaks are those identified as particular proteins.* (C) *Subject B, day 57.* (D) *Subject G, day 48.*

quadrants to facilitate female versus female, male versus male, and female versus male comparisons, shows the number of such nonoverlapping peak heights in each subject-subject comparison. The fact that a number greater than 0 appears in each of the subject-subject comparisons signifies that individualization by the criterion mentioned above is possible. It should be mentioned, however, that when one or more peaks is usually present in one individual and always absent in another, and when the comparison is fully based on such peaks (when in Table 2 the first number and the number in parentheses are the same) individualization cannot be certain if in some runs the peak is missing in the individual when it is usually present. For example, the distinction between Subjects A and I depends fully on the presence of Peak 20 in the former and its absence in the latter. This proved to be true in only eight of the ten runs, so it can be said that there is a 20% chance that in any single run the two subjects could not be distinguished. The only other similar situation was the comparison of Subjects D and I, where there was also a 20% chance for nondistinction (see below for other aspects of this comparison).

Several other points of interest should be made concerning the data in Table 2. By determining the average number of peaks with nonoverlapping ranges in the different quadrants one can gain an estimate of the relative degrees of variation among females, among males, and between females and males. For example, 25 female-male comparisons involved 109 nonoverlapping peaks for an average of 4.36; corresponding values for female-female and male-male comparisons (excluding self-self comparisons) were 3.2 and 1.9, respectively. Thus, the greatest variation was, as would be expected, between sexes; what was not necessarily expected was considerably more variation among females than among males. Finally, it should be mentioned that the only

TABLE 1—*Means and ranges of precipitin peak heights in all subjects.*

Peak	Subject A	B	C	D	E	F	G	H	I	J
1 Mean	15.6	15.6	16.2 [a]	14.9	16.3	15.3	14.3	13.5	14.9	14.5
1 Range	14–17	13.5–17	16–21	13.5–17	15–17	12.5–17.5	13–15	12–14.5	13.5–16	12.5–16
2 Mean	25.4 [a]	29.6	32.6	27.8	28.7	27.6 [a]	31.8	30.7 [a]	26	23.6 [a]
2 Range	21.5–28.5	25.5–36.5	28.5–37.5	26–29.5	23.5–31.5	25.5–29.5	29–34.5	28–33.5	24.5–28.5	21.5–26.5
3 Mean	38.2	34.8	34.7	36.1	32.2	39.2	35.8	34.3	35.8	33
3 Range	31–44	31–37.5	29.5–41.5	32.5–40	23.5–38	33.5–43.5	31.5–40	30–41	31.5–39	30.5–35.5
4 Mean	25.5	27.6	19.9	21.6	23.5	23	19.4	19.1	21.9	21.2
4 Range	22–29	25–30.5	17–24	19.5–25	20.5–26	20–25	17.5–21.5	17.5–23	20–24	19–24
5 Mean	17.2	17.7	17.6	17.9	17.8	17.8	16.4	15.4	16	16
5 Range	15.5–19	16.5–20	14.5–21.5	16–19.5	15.5–19	14.5–20.5	15–17.5	13.5–18	14.5–17.5	14.5–18
6 Mean	16.1	16.5	16.2	14.6	16.7	15.3	14.8	14.6	14.9	13.4
6 Range	13.5–18	15.5–18.5	14–18.5	13–16	14.5–17.5	13.5–17	14–16	13–17.5	14–16.5	12–14.5
7 Mean	16	15	14.3	15	13.1 [a]	15.8	14.7	13.4	14.6	14.1 [a]
7 Range	13.5–17	13–18	12.5–16.5	13.5–17	12.5–14	14–18	13.5–16	12.5–15.5	13.5–16	13–15.5
8 Mean	16.8	21.1	20.2	15.6 [a]	22.4	. . . [b]	. . . [b]	15.2 [a]	15.1 [a]	13.8 [a]
8 Range	15.5–18.5	19–23	17–25	14.5–17.5	21–24	. . . [b]	. . . [b]	14.5–17.5	14–16	13–16
9 Mean	16.2	26.4	20.2	14.6	26.3	15.1	14.1	14.4 [a]	14.7 [a]	14.1 [a]
9 Range	14–17	22.5–29	16–25.5	13.5–16.5	23–29	13–19.5	13–15.5	13–17	13.5–16.5	12.5–15.5
10 Mean	18.3	21.6	26	. . . [b]	20.9	16.2	15.1 [a]	13.7	15.8 [a]	14.6 [a]
10 Range	16–20	20–23	21–31.5	. . . [b]	18.5–22.5	13.5–19.5	14–16.5	12.5–16	14.5–17	13–16
11 Mean	14.9	17.2	16.4	12.3 [a]	17	14	13.2	13.3	13.8	. . . [b]
11 Range	12–17	15.5–18.5	13.5–20.5	11.5–14.5	11–19	12.5–16	11–15	11–16.5	12–16.5	. . . [b]

12 Mean	32	22.8	23	22.1	23.3	. . .[b]	19.8	22.8	28.5	26.4
12 Range	25 –40	20.5–27	17.5–27	17.5–25.5	20 –25.5	. . .[b]	17 –25	20 –29	23 –37.5	22 –31.5
13 Mean	12.9	12.7	12.2[a]	. . .[b]	11.5	11.5[a]	12.5	10.9[a]	13.2[a]	12
13 Range	11.5–15	12 –14	11.5–13	. . .[b]	10.5–13	10.5–13	12 –13.5	10 –12.5	12 –14.5	10.5–13.5
14 Mean	21.3	22.3	22.5	19	22.8	20.6	18.7	18.3	17.9	16.6
14 Range	16 –27	19.5–24.5	18.5–28	16 –21	20 –26	16.5–23	15.5–21.5	16.5–22.5	13.5–21	14.5–18
15 Mean	19.1	19	21.5	16.4	20.1	17.7	16.4	16.9	16.8	15.5
15 Range	14 –22.5	17 –20	18 –26	14.5–18	18 –23	14 –20	14 –18.5	15 –20.5	13 –20	14 –16.5
16 Mean	11.9[a]	12.6[a]	12.6[a]	12.4[a]	12.7	12.5	12.2[a]	11.9	12	12.6[a]
16 Range	11 –13	11.5–13.5	11 –13.5	11.5–13	12 –13.5	11.5–13.5	11 –13	11 –13.5	11 –13	10.5–14
17 Mean	11.9	11.2	10.8	11.9	11.3	11.6	10.8	10.1	11.1	10.9
17 Range	10.5–13	10 –12.5	10 –11.5	11 –12.5	10.5–12	10.5–12.5	9.5–11.5	9.5–11.5	10.5–12	9.5–11.5
18 Mean	11.4	10.4[a]	10.4[a]	10.5	10.4[a]	9.8	10.4	9.7	10.4	9.5
18 Range	9.5–14	9.5–11	9.5–11.5	9.5–11.5	9.5–12	8.5–11	9 –11.5	9 –11	9 –12	8.5–10
19 Mean	10[a]	10.1	9.9	9.1[a]	10	10.6[a]	9.6[a]	9.1[a]	9.6	9.1[a]
19 Range	9 –11	9.5–10.5	9 –11.5	8.5– 9.5	9 –11.5	9.5–11.5	8.5–10.5	8.5–10	8.5–10.5	8 –10.5
20 Mean	9[a]	9.4	. . .[b]	. . .[b]	. . .[b]	8.9	. . .[b]	8.8	. . .[b]	. . .[b]
20 Range	8 –10	9 – 9.5	. . .[b]	. . .[b]	. . .[b]	8– 9.5	. . .[b]	8 – 9.5	. . .[b]	. . .[b]
21 Mean	45.5	43.1[a]	45.9[a]	42.1[a]	. . .[b]	47.1[a]	42.8[a]	44.2[a]	45.4[a]	39.2
21 Range	42 –49	39 –50.5	37.5–52.5	35.5–48.5	. . .[b]	39.5–51	38 –49	42.5–46.5	38.5–54.5	34 –43
22 Mean	37	36.2	40.3	33.3	40.9	36.1[a]	32.4[a]	31.6[a]	36.6[a]	30.4[a]
22 Range	34 –41	31.5–40	30.5–49.5	28.5–36.5	35.5–45	29.5–42	28.5–36.5	30 –34.5	31.5–41	28 –32

[a] Peak resolved in at least 6 but less than 10 runs.
[b] Peak not resolved in 10 of 10 runs.

TABLE 2—*Distinction between subjects on the basis of nonoverlap in ranges of heights of precipitin peaks.* [a]

		Females					Males				
		A	B	C	D	E	F	G	H	I	J
Female	A	...	3(0)	2(1)	3(2)	4(2)	3(2)	4(2)	2(0)	1(1)	4(2)
Female	B	3(0)	...	1(0)	7(2)	2(2)	5(2)	5(1)	4(0)	5(0)	9(2)
Female	C	2(1)	1(0)	...	4(2)	1(1)	3(2)	4(1)	2(0)	4(0)	8(1)
Female	D	3(2)	7(2)	4(2)	...	5(3)	4(3)	3(3)	3(3)	2(2)	4(3)
Female	E	4(2)	2(2)	1(1)	5(3)	...	6(4)	4(2)	7(2)	4(1)	9(2)
Male	F	3(2)	5(2)	3(2)	4(3)	6(4)	...	2(2)	2(2)	2(2)	4(4)
Male	G	4(2)	5(1)	4(1)	3(3)	4(2)	2(2)	...	2(2)	1(0)	2(1)
Male	H	2(0)	4(0)	2(0)	3(3)	7(2)	2(2)	2(2)	...	1(0)	2(1)
Male	I	1(1)	5(0)	4(0)	2(2)	4(1)	2(2)	1(0)	1(0)	...	1(1)
Male	J	4(2)	9(2)	8(1)	4(3)	9(2)	4(4)	2(1)	2(1)	1(1)	...

[a] The first number is the total number of peaks with nonoverlapping ranges in subject-subject comparison; the number in parentheses is the number of peaks absent in the pattern of one subject and present in the other.

siblings involved in the study (Subjects I and J are brothers) and the only non-Caucasian (Subject D is a Persian female) provided some surprising results: Subject I could be more easily distinguished from his brother than from two of the females, one of whom was Subject D.

The histogram in Fig. 2 shows the number of times the different precipitin peaks were of value (they had nonoverlapping ranges) in making overall distinctions and distinctions among males, among females, and between males and females. Peaks of value in one or no comparisons are not shown. As can be seen, Peaks 8, 9, and 10 were

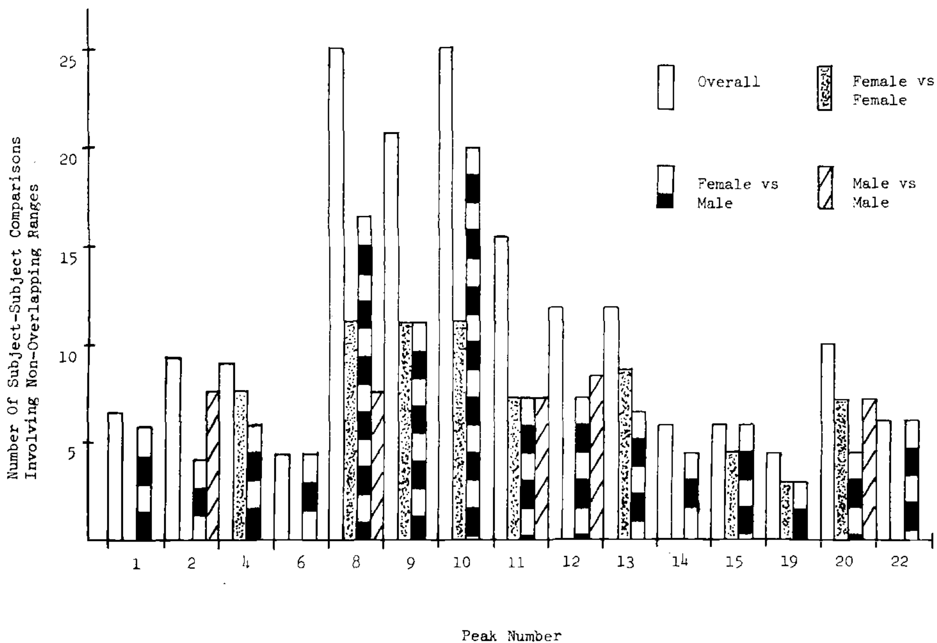

FIG. 2—*Relative values of different CEID peaks in distinguishing females from females, males from males, females from males, and overall. Those peaks of value in one or no distinctions are not shown.*

most valuable in overall, female-male, and female-female distinctions. Peaks 2, 8, 11, 12, and 20 were about equal and the only peaks of value in making male-male distinctions. Two of them (2 and 12) were of no value in female-female distinctions. Peaks 3, 5, 16, 17, 18, and 21 had overlapping ranges in all comparisons and therefore were of no value in individualization.

To assess CEID as a means for distinguishing female from male bloodstains, Students' t tests were done on each of the 22 peaks. As shown in Table 3, there were statistically significant sexual differences in the heights of Peaks 1 (α_1-lipoprotein), 5 (Gc globulin), 6, 8, 9, 10 (ceruloplasmin), 14 (hemopexin), 15 (transferrin), and 18 (immunoglobulin A).

Discussion

Until recently virtually all research on the serologic characterization of bloodstains has been directed toward improvement and extension of typing according to the various groups of erythrocyte antigens. We chose to depart from this approach because, aside from its unquestioned value, it lacks the potential for attainment of what is probably the primary goal of forensic serology—the "fingerprinting" or individualization of human bloodstains. This is so because typing detects only qualitative differences, that is, differences in kinds of antigens, and no human is unique in respect to the known erythrocyte antigens.

Clearly, if individualization of bloodstains is to become a reality a technique must detect not only differences in kind but also in quantity. This point is illustrated by the results in Fig. 1; although some CEID patterns can be distinguished on the basis of qualitative differences (absence of certain peaks in some patterns), it is the quantitative aspects (differences in peak heights) that allow one to conclude that each pattern is different from all others.

TABLE 3—*Tests for significant differences between females and males in precipitin peak heights.*

		Female		Male			
Peak	Antigen	Mean	SD	Mean	SD	t Value	P Value
1	α_1-lipoprotein	15.72	0.563	14.50	0.678	3.095	<0.02
2	prealbumin	28.82	2.629	27.40	3.637	0.708	NS [a]
3	albumin	35.20	2.192	35.62	2.318	0.294	NS
4	α_1-antitrypsin	23.62	3.054	20.92	1.657	1.737	NS
5	Gc globulin	17.64	0.270	16.32	0.901	3.138	<0.02
6	not identified	16.02	0.829	14.60	0.718	2.896	<0.02
7	not identified	14.68	1.071	14.52	0.882	0.258	NS
8	not identified	19.22	2.897	14.70	0.781	2.570	<0.05
9	not identified	20.74	5.513	14.48	0.427	2.532	<0.05
10	ceruloplasmin	21.70	3.199	15.08	0.988	4.438	<0.01
11	not identified	15.56	2.033	13.58	0.386	1.899	NS
12	haptoglobin	24.64	4.138	24.38	3.853	0.098	NS
13	not identified	12.33	0.624	12.02	0.887	0.579	NS
14	hemopexin	21.58	1.548	18.42	1.452	3.329	<0.02
15	transferrin	19.22	1.870	16.66	0.802	2.813	<0.05
16	not identified	12.44	0.321	12.24	0.305	1.011	NS
17	α_2-macroglobulin	11.42	0.476	10.90	0.543	1.609	NS
18	immunoglobulin A	10.62	0.438	9.96	0.416	2.443	<0.05
19	not identified	9.82	0.409	9.60	0.612	0.668	NS
20	not identified	9.20	0.283	8.85	0.071	1.698	NS
21	not identified	44.15	1.840	43.74	2.990	0.237	NS
22	not identified	37.54	3.121	33.42	2.773	2.206	NS

[a] NS = not significant.

Unfortunately, the necessary dependence of individualization on quantitative differences brings with it a complicating factor which, until now, has not been appropriately considered. Due to normal physiologic variation, the quantities of a single person's serum proteins might be different on different occasions, so that individualization would require a demonstration that such variations were different and distinguishable from those of some other person. Ordinarily, an acceptable demonstration that the variation within individuals is less than or different from variation among individuals is based on calculations of statistical significance. However, we felt a more appropriate approach for this study would be to ascribe significance to a precipitin peak only if its range in height, over several determinations, was completely different in subject-subject comparisons. Thus, for example, even though there was a highly significant statistical difference ($P<0.0005$, Students' t test) in the height of Peak 1 in comparing Subjects C and G, we considered it to be not significant for individualization because the ranges overlapped somewhat.

As expected, when our more stringent criterion for individualization was used in all the subject-subject comparisons (Table 2) there were considerably fewer peaks with significant differences than would seem to be the case if one compared only single patterns from each individual (Fig. 1). Nevertheless, it is especially noteworthy that individualization was still possible among a group of subjects that was, in comparison with the general population, relatively homogenous in age, race, and state of health.

The dependence of each subject-subject distinction on a small number of peaks suggests that, in the overall population, some serum proteins might never show sufficient differences among individuals to be of value. If this proved to be the case, one could identify such antigens and selectively eliminate them, or more easily their corresponding antibodies, from the assay system with resulting CEID patterns that were simpler and therefore easier to interpret than those obtained in this and earlier studies [*24, 25, 29*]. The analysis of our data indeed suggests that a sizable number of the human serum proteins show little or no significant differences among individuals; 6 of the 22 antigens showed no significant differences in any of the 65 subject-subject comparisons, and an additional seven antigens were significant in only five or less of the comparisons. Perhaps of even greater importance was the small number of significantly different proteins in same-sex comparisons, especially those among males where only five antigens were of value in the 20 subject-subject comparisons (Fig. 2). Thus, what is probably the most frequently encountered forensic-serologic problem—distinction among male suspects—could conceivably be solved by use of a simplified, five-peak (2, 8, 11, 12, and 20), CEID procedure.

Our attempts to identify each of the 22 peaks as particular serum proteins, although only partially successful, were adequate to suggest that future patterns could be simplified, without loss of value, by selective elimination of antibodies to (a) albumin, Gc globulin, α_2-macroglobulin, and immunoglobulin A in any comparison, regardless of sexual origin, (b) α_1-lipoprotein, α_1-antitrypsin, ceruloplasmin, hemopexin, and transferrin in known male-male comparisons, and (c) α_1-lipoprotein, prealbumin, haptoglobin, and hemopexin in known female-female comparisons. We emphasize that the above statements refer specifically to the lack of value of certain antibodies in establishing individuality; some of them are not without value in making probability statements concerning the sexual origin of a stain (discussed below). A disappointing aspect of this part of the study was the failure to identify Proteins 8, 11, and 20, three of the most valuable distinguishing antigens overall and within the same sex.

Although this investigation was not intended nor designed to determine whether the kinds and amounts of various serum antigens are under the same genetic controls as RBC blood types, the limited data we have on relatives provides no indication of such; the brothers (Subjects I and J), even though they were different in only one protein, could nevertheless be distinguished from each other as easily as the subjects in ten of

the other comparisons, and easier than in comparisons which involved one of the brothers and two different females (Table 2).

In considering the practicality of adding CEID to the routine procedures of forensic serology we have identified in this study a serious, but not insurmountable, problem. The present lack of knowledge concerning the normal range of concentrations of serum proteins in individual humans dictates that such a range be established, for any suspect, before it can be said with any certainty that a bloodstain did or did not come from that suspect. This, in practice, would require multiple blood donations by the suspect over a period of time; a prospect that, needless to say, is not appealing or in some cases not practical, possible, or legal. It should be strongly emphasized, however, that this problem is not one of technique but of lack of information. We hope that the increased application of CEID within and outside forensic studies [*25, 29*] will lead to the establishment of normal values in the population at large so that, in the future, the considerable discriminatory power of the technique can be used to advantage in runs on single bloodstains.

Aside from our evaluation of CEID as a means for individualization, we considered it of interest to examine the technique's ability to distinguish male from female bloodstains. Our interest was prompted by (a) the obvious forensic value of such a distinction, (b) the lack of any other reliable means for determining the sexual origin of a stain (to our knowledge, the best available procedure is based on Y chromosome fluorescence and yields positive results only 55% of the time in 48-h stains from males [*27*]), and (c) the finding by Clarke and Freeman [*25*] that CEID reveals significant male-female differences in certain serum proteins (α_1-lipoprotein, ceruloplasmin, and α_2-macroglobulin). As can be seen in Table 3, we found statistically significant male-female differences in 9 of the 22 bloodstain antigens, 6 of which were identified as α_1-lipoprotein, Gc globulin, ceruloplasmin, hemopexin, transferrin, and immunoglobulin A. These results are consistent with those of Clarke and Freeman in showing significant sexual differences in α_1-lipoprotein and ceruloplasmin but are inconsistent with their finding of significant differences in α_2-macroglobulin and no significant differences in Gc globulin, hemopexin, and transferrin. Whether the inconsistencies are due to their use of fresh serum as contrasted with our use of eluted bloodstain, to differences in sample size or differences in subject ages, or to unrecognized variables cannot be decided. Whatever the case, our results along with theirs strongly argue for serious consideration of CEID patterns, especially those peaks produced by α_1-lipoprotein and ceruloplasmin, as a way to judge the probability of a stain coming from one sex or the other.

Summary

Crossed electroimmunodiffusion (CEID) was evaluated as a means for individualizing human bloodstains by studying variations within and among individuals in 22 serum antigens in ten subjects over a four-month period. The extent of variation within an individual was determined by making CEID runs on bloodstains obtained on ten different occasions and measuring the precipitin peak heights produced by each of the 22 antigens. When the range in height of any particular peak was completely different in subject-subject comparisons, the peak was judged to be of value in individualization. By this criterion, each of the ten subjects could be distinguished from all others (65 subject-subject distinctions), but in most cases the distinction had to be based on differences in 5 or less of the 22 antigens. The antigens of value in distinguishing among males were largely different from those of value in distinguishing among females. Overall, the antigens of greatest value in individualization were 8, 9, 10, and 11. Only one of these (10, ceruloplasmin) could be identified as a particular serum protein.

In other studies on CEID as a means for establishing the sexual origin of a stain there was found a statistically significant female-male difference in the heights of Peaks 1 (α_1-lipoprotein), 5 (Gc globulin), 6, 8, 9, 10 (ceruloplasmin), 14 (hemopexin), 15 (transferrin), and 18 (immunoglobulin A).

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