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Attempts to Determine the Lewis Phenotypes of Dried Bloodstains

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ABSTRACT: Whole bloods and body secretions are routinely examined for their Lewis factors; however, no method for typing dried bloodstains in this system currently exists. This research investigated the use of absorption elution. Antigoat immunoglobulins were used to aid agglutination of indicator cells.

KEYWORDS: pathology and biology, antigen systems, blood, immunoglobulins, Lewis antigens, antigoat immunoglobulins, bloodstains

The Lewis system consists of two antigens, Le^a and Le^b, which together divide individuals into three common red cell phenotypes, Le(a−, b−), Le(a+, b−), and Le(a−, b+). These antigens are not indigenous to the red cell membrane, but instead are adsorbed onto the red cells from the plasma. Production of the Lewis antigens is not only dependent on the Lewis genes, but also on the secretor genes and the Hh genes.

The Lewis blood grouping system is used in the forensic science laboratory to indicate the ABO secretor status of an individual. Since whole blood samples are routinely typed for their Lewis phenotype, it would be advantageous to be able to determine the Lewis phenotype of a dried bloodstain and apply accepted population frequencies to the results.

There is very little in the literature concerning the Lewis typing of dried stains. Pereira and Martin [*1*] described the determination of Lewis secretions of dried stains of body fluids, particularly in saliva, but did not discuss dried bloodstains. Davie [*2*] investigated Lewis typing of dried bloodstains by absorption-inhibition on microtitre plates, and reported that they were most difficult to type, with stains over two months old being untypable.

Purpose

The purpose of this research was twofold:

(1) to develop a method for Lewis typing of dried bloodstains and
(2) to determine the persistence of the Lewis substance in dried bloodstains.

Development of a method for typing dried bloodstains in this system seemed desirable because:

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1. Population frequencies for Lewis phenotypes are already published and are widely accepted.

2. In the detection of protein markers in bloodstains, the structural conformation of the entire protein molecule must remain intact. Enzyme markers must remain enzymatically active in stains to be visualized following electrophoresis.

The Lewis system is an immunological marker. Immunological detection of genetic markers requires only that the immunological determinant of the genetic marker molecule remain immunologically active in a dried stain, thereby offering the possibility of stability and detection over a longer time than the protein/enzyme markers.

Materials

Antisera

Lewis antisera [*3*] (goat) was obtained from Ortho Diagnostic Systems, Inc., and was used neat. The rabbit antigoat immunoglobulins (composition unknown) were obtained from Accurate Chemicals, Westbury, NY. The swine antigoat immunoglobulin macro (IgM) was obtained from Nordic Immunological Laboratories, El Toro, CA.

Indicator Cells

Le(a+, b−) and Le(a−, b+) indicator cells were prepared fresh daily from Ortho Resolve panel cells. Additional indicator cells were obtained by finger prick from Type O, Le(a+, b−) and O, Le(a−, b+) donors.

Samples

Bloodstains were prepared from whole blood obtained by finger prick from laboratory staff and were prepared on clean white cotton cloth. Additional blood samples were obtained from whole bloods from criminal cases.

Methods

Preparation of Indicator Cells

Two Group O panel cell suspensions, one Le(a−, b+) and the other Le(a+, b−) were washed three times in isotonic saline. The cells were then treated with trypsin for 15 min at 37°C, followed by three more saline washes to remove any excess trypsin. A 0.5% suspension of the cells was made up in isotonic saline.

The trypsin used consisted of a stock solution and a buffer solution. The trypsin stock solution was prepared by adding 2.5 g of trypsin powder to 10 mL of 0.05*N* hydrochloric acid. This mixture was placed in a refrigerator and shaken at intervals. After 24 h, the mixture was centrifuged and the supernatant collected.

Of this stock solution, 0.2 mL was then added to 1.8 mL of the trypsin buffer solution. This buffer solution was a 0.1*N* phosphate buffer, pH7.7, consisting of 6.28-g sodium phosphate dibasic and 0.78-g potassium phosphate monobasic in 500 mL of water.

Typing of Liquid Blood

Liquid blood samples were examined for their phenotype by a capillary tube method [*4*]. The cells were trypsinized in the same manner as the indicator cells. A 35% cell suspension in isotonic saline was prepared. Approximately 10 μL of Lewis antiserum was drawn into a

20-μL capillary pipette. Approximately 5 μL of the cell suspension to be analyzed was drawn into the same capillary pipette, which was then inverted and inserted into a clay base. After 5 to 10 min the tubes were read macroscopically for agglutination.

Typing of Dried Bloodstains

Absorption elution [5] was used to type dried bloodstains. Trypsinized indicator cells were employed to aid in agglutination, as tests with nontrypsinized cells failed to agglutinate. What follows is a description of the method used at the outset of this project.

Approximately 2 by 2-mm squares of bloodstained cloth were added to two test tubes. One drop of undiluted anti-Lewis A was added to one tube, one drop of anti-Lewis B to the other. The samples were allowed to absorb overnight at 4°C, after which the excess antisera was removed by aspiration, and the stains subjected to six 20-min washings to remove any unabsorbed antibodies. The washings consisted of adding cold saline to the tubes and allowing them to stand at 4°C for 20 min. After withdrawing the final saline wash, two drops of isotonic saline were added to each tube, and the samples were allowed to elute for 15 min at 56°C in an incubator. The eluate was withdrawn from the tubes, placed on glass plates, treated with the appropriate indicator cells, rotated for 10 min, and read microscopically for agglutination.

At different points in the research, the following variations were attempted:

1. The absorption temperature was changed to 25°C with everything else remaining constant.
2. The stains were fixed with methanol.
3. The stains were fixed with ethyl ether.
4. Instead of soak-washing, the samples were vortex-washed until foaming ceased.
5. Four 1-cm threads were tested using the Howard-Martin elution technique and trypsinized indicator cells.
6. After completion of the washing step, the samples were eluted in microcentrifuge tubes. The indicator cells were added directly to the tubes, which were then centrifuged for 30 s. The eluate/indicator cell mixture was placed on glass plates and read microscopically for agglutination.
7. Antigoat immunoglobulins were used at various points in the above procedures, in case the amount of Lewis antibodies eluted from a bloodstain might only be enough to sensitize the indicator cells, but not agglutinate them. The antigoat immunoglobulins would serve to agglutinate such sensitized cells by bridging them through the attached Lewis antibodies, as in the direct Coombs test.

Absorption inhibition was also investigated for comparative purposes. For this technique, 4-mm^2 pieces of bloodstained cloth were placed in test tubes, and treated with one drop of diluted Lewis antisera. The proper dilution of antisera was the highest dilution still affecting a +4 agglutination of indicator cells. For the antisera used in this investigation, the dilution was 1/20. The samples absorbed overnight at 4°C, after which the unabsorbed antibodies were removed, treated with the appropriate indicator cells, allowed to react at room temperature, centrifuged for 30 s at 3400 rpm, and examined microscopically for agglutination.

Tests with Antigoat IgM

The rabbit antigoat immunoglobulins originally used in this project induced the formation of a white, gelatinous precipitate. This precipitate made observation of the eluate/indicator cell mixture difficult and caused false agglutinations.

In a successful attempt to avoid these problems, swine antigoat IgM was employed in a series of tests. These antibodies are produced in swine to react with the IgM fraction of goat globulins.

In these tests, indicator cells were titred against the appropriate Lewis antisera, allowed to react, centrifuged, and read. Next, the antigoat IgM antisera was added to attempt to increase agglutination in the higher dilutions of Lewis antisera. The cells were again allowed to react, centrifuged, and read.

Results

Absorption Elution

After attempting all of the variations described above (Table 1), the results of the different methods were tabulated. None of the methods was successful. Each of the variations attempted suffered from a lack of reproducibility. The results are summarized in Table 2.

Because of the high rate of false identifications, blind studies were not attempted.

Absorption Inhibition

The absorption inhibition method also suffered from a lack of reproducibility. The results are summarized in Table 3.

Antigoat IgM Tests

The antigoat IgM failed to enhance agglutination significantly.

Discussion

As a result of this study the conclusion has been reached that neither absorption elution nor absorption inhibition, as attempted, is a reliable technique for the Lewis typing of dried bloodstains.

Because the number of Lewis antigens on the red blood cell are few in comparison to the ABO antigens, it could be that the method is not sufficiently sensitive to detect Lewis anti-

TABLE 1—*Comparison of variations used.*

Variation	Percent Correct	Percent Incorrect
Coombs test with centrifugation	47.9	52.1
Absorption inhibition	44.8	55.2
Vortex washing	31.4	68.6
Fixed samples	29.4	70.6
Modified Howard-Martin	28.6	71.4
Basic absorption elution	21.5	78.5
Coombs test with rotation	19.0	81.0
Absorption at 25°C	11.1	88.9

TABLE 2—*Summarized results from absorption elution experiments.*

	Observed Phenotype			
Actual Phenotype	a+b−	a−b+	a+b+	a−b−
Le(a+b−)	21	4	9	32
Le(a−b+)	14	31	30	73
Le(a−b−)	7	3	2	27

TABLE 3—*Summarized results from absorption inhibition experiments.*

Actual Phenotype	Observed Phenotype			
	a+b−	a−b+	a+b+	a−b−
Le(a+b−)	25	1	4	14
Le(a−b+)	6	7	12	10
Le(a−b−)	7	1	3	15

gens in bloodstains reliably, causing false negative agglutinations. False positive agglutinations could have been the result of poor washing techniques. Proteolytic treatment of indicator cells can often lead to indiscriminate agglutination (false positives).

Stain age had an erratic effect on the ability to type bloodstains correctly. Some stains up to four months old were consistently typed correctly, while some stains as little as two weeks old could not be typed.

For further research in this area, the investigation of either radioimmune assay or enzyme linked immunosorbent assay (ELISA) is recommended. Apparently, following elution there is such a small amount of antibody present that these techniques would be necessary to detect their presence. Also, further research might be required to develop a better understanding of the Lewis system and what happens to it in dried stains.

Summary

Absorption elution and absorption inhibition were explored as possible techniques for Lewis typing of dried bloodstains. Results indicated the modifications of these methods tested are not reproducible for the Lewis system.

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