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USE OF ENZYME-TREATED CELLS IN GROUPING DRIED BLOODSTAINS*

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The grouping of dried bloodstains is a difficult, tedious, and time-consuming procedure which requires proper preparations, techniques, and patience, coupled with ability and experience, to evaluate properly results of tests conducted. It has been shown that a bloodstain may undergo serious alterations in its composition between the time of its deposit and the time of examination in the laboratory. It is imperative for the forensic investigator to be acquainted with the difficulties that may be encountered. It follows that the forensic investigator should have at his command as many techniques as possible to cope with the various problems that arise in attempting to group dried bloodstains.

In grouping dried bloodstains, the expert usually selects one of two avenues of investigation: detecting the presence or absence of isoagglutinins in a bloodstain or detecting the presence or absence of agglutinogens in a bloodstain. Detecting the presence or absence of isoagglutinins may be accomplished by the Lattes or Crust Method (1). Detecting the presence or absence of agglutinogens is usually accomplished by the Absorption Inhibition (2), Absorption Elution (3), or Mixed Agglutination (4) tests. Whenever possible, both methods, detecting the isoagglutinins and detecting the agglutinogens, should be accomplished.

In many instances, notably in dried, aged stains, the isoagglutinins are altered and may be partially or completely destroyed. Isoagglutinins are known to be affected by heat. Sunlight and humidity are also destructive factors. As a general rule, it is suggested that isoagglutinins are relatively labile whereas agglutinogens are far more resistant (5).

Experience in our laboratory with dried bloodstains, of known blood groups, aged ten days or

more, show the isoagglutinins in many cases to be partially to totally absent. Therefore, in grouping bloodstains we have relied primarily on the Absorption Elution (3) and Mixed Agglutination (4) tests. Although these tests are reliable and accurate, in many cases, a high degree of sensitivity is lacking. Because of the relative instability of the isoagglutinins in dried bloodstains and the desire to obtain greater sensitivity in detecting agglutinogens present in bloodstains, it was decided to investigate the use of enzyme-modified cells in detecting absorbed antibodies by means of the Absorption Elution method.

Studies have shown that enzyme-treated cells alter the A and B factors so that agglutination is of better avidity and titration values higher than in saline (6). Such cells serve as sensitive indicators of blood group antibodies. However, the use of certain enzymes, notably trypsin and ficin, quickly pointed out inherent limitations: the tediousness of pretreating the red cells which entails washing and preparation of the treated cells, and frequently non-specificity in those sera containing cold antibodies.

Research conducted on a group of enzymes derived from the pineapple stem and known as bromelin has shown that this enzyme may be used to demonstrate erythrocyte antibodies (7). It was observed that isotonic solutions of bromelin would induce rapid agglutination when mixed directly with antibodies containing serums and erythrocytes. Of special interest was the sensitivity of the "immediate bromelin" test at room temperature. This test was performed using 0.1 ml of the antibody serum, 1 drop of the solution of bromelin, and 1 drop of 4% isotonic saline suspensions of unwashed erythrocytes. The reagents were mixed in a tube, immediately centrifuged for 1 minute at 1000 rpm and read macroscopically. The use of bromelin thus eliminated the necessity of time-consuming pretreatment of erythrocytes, lengthy

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washings, and a slower reaction time. Bromelin is also a relatively stable enzyme and maintains potency for approximately 30 days when stored at 4°C. Further treatment with 0.1% sodium azide increases the stability range to over two months.

The use of proteolytic enzymes to determine blood groups of dried stains has previously been reported. Inella and Redner demonstrated the use of ficin modified cells in detecting the presence of isoagglutinins in bloodstains (8). The results obtained by the use of ficin-modified cells were corroborated by tests using normal saline cell suspensions with the Absorption Inhibition tests.

As stated previously, our objective was to evaluate critically the use of bromelin as a sensitizing enzyme for determination of 'ABO' blood groups of dried stains. Fifty-five stains of known blood groups (A, B, AB, and O) were prepared on backgrounds of cotton, wool, synthetic fabrics, wood, glass, and metal. At the time tests were conducted on these stains, they ranged from 10 to 30 days in age. The amount of stained material ranged from a few fibers to areas approximately 2 cm square. In the case of glass or metal, 100-150 mg of scrapings were removed and tested. Each stain, with the exception of stains of group AB, was subjected to hemagglutination tests for isoagglutinins. Because of the lack of isoagglutinins in bloodstains of group AB, these stains were tested by the Absorption Elution technique (3) using normal saline cell suspensions and bromelin modified cell suspensions, as were bloodstains of group A, B, and O. The modified Absorption Elution technique of Marigo, Fiori, and Benciolini (3) was selected with certain further modifications; namely, the use of anti-H lectin to detect the H antigen and the reduction of incubation time for six hours to two hours.

REAGENTS

1. Human immune anti-A and anti-B sera (titer 256)¹
2. Bromelin Reagent¹
3. Anti-H lectin [*Ulex Europaeus*] (titer $\frac{1}{64}$)²
4. Fresh 2% suspensions of A, B, and O cells (5 cc of each).
5. Methyl Alcohol

The bromelin reagent may be prepared as follows:

¹ Supplied by Michael Reese Research Foundation, Chicago, Illinois.

² Supplied by Hyland Laboratories, Los Angeles, California.

A. Sorenson's Buffer: (pH 5.5 - 0.15 M)

- 1) 2.04 grams KH_2PO_4 + 100 cc distilled water
 - 2) 2.13 grams Na_2HPO_4 + 100 cc distilled water
- Add 95 cc of solution (1) to 5 cc of solution (2)

B. Bromelin reagent

0.5 grams of concentrated bromelin powder
+ 90 cc of saline + 10 cc of Sorenson's Buffer

The reagent may be stored in aliquots (3 ml) at 4°C and removed approximately one hour before use and allowed to reach room temperature.

PROCEDURE

1. The test material (stained fibers, cloth, wood, or blood scrapings) is placed directly in a Petri dish. Unstained test material is placed in a separate Petri dish and will serve as a control. The unstained material will be subjected to the same procedure as the stained material throughout the test. In the case of fibers, cloth, or wood, an unstained area immediately adjacent to the stain is selected for control purposes. Where the stained material is blood scrapings, saline solution is used as a control.

2. The test material in the Petri dish is examined under the stereobinocular microscope and any foreign debris removed.

3. Enough methyl alcohol to cover the fibers, cloth, or wood completely is introduced. In cases where the test material is blood scrapings, enough methyl alcohol is introduced so as to totally immerse the scrapings. The Petri dish is covered, and the test material allowed to absorb the alcohol for approximately 15 minutes.

4. At the end of the denaturation period, any excess methyl alcohol is drained off by Pasteur pipette and the test material allowed to air dry.

5. Approximate equal portions of the test material are placed in three 6 x 60 mm test tubes, suitably marked A, B, and H.

With fibers, cloth, or wood division of the test material into approximate equal parts and transferral to the test tubes is relatively simple. Where the test material is blood scrapings, the scrapings are carefully collected on the lip of the Petri dish with the aid of a stainless steel spatula while holding the Petri dish inclined at an oblique angle. The respective test tube is held in a holder beneath the Petri dish with the top of the test tube touching the lip of the Petri dish. Approximate equal

amounts of blood scrapings are teased into each of the three test tubes by aid of the spatula. Careful attention to detail will prevent any appreciable loss of test material.

6. If the test material is fibers, cloth, or wood, equal amounts of antisera, enough to completely saturate the stain, are introduced: anti-A to tube A; anti-B to tube B; and anti-H to tube H. Where the test material is scrapings, enough antisera is introduced to each tube so that the test material is immersed.

7. The test material and antisera are then incubated at 4°C for two hours. Some workers prefer to perform absorption at room temperature; however, our experience has shown that we obtain poorer results by incubating at room temperature. We agree with Fiori, Benciolini, and Marigo (3), whose absorption elution technique we followed, that incubation at 4°C produces more complete antibody absorption by the antigens and consequently better results are obtained in detecting absorbed antibodies.

8. At this time a bromelin aliquot is removed from the refrigerator and allowed to reach room temperature.

9. Approximately twenty minutes before the completion of incubation, 2 ml of the normal cell suspensions (indicator cells) A, B, and O are modified by the bromelin reagent. This is accomplished by adding 2 ml of bromelin reagent to 2 ml of A, B, and O cells, respectively. The remaining normal cell suspensions will be utilized later in the procedure.

10. At this point control tests are performed, which consists of adding 0.1 ml of bromelin-modified A cells to 0.1 ml of anti-B serum in a 6 x 60 test tube. The contents of the tube are centrifuged at 1000 rpm for one minute and examined macroscopically for agglutination. No agglutination must occur. This procedure is repeated utilizing bromelin-modified B cells and anti-A serum. Again, no agglutination must occur. This control is inserted to test for the possibility of reactions caused by the bromelin per se.

11. The test material and antisera are removed from the refrigerator and the contents of each tube washed six or seven times with ice cold saline. Washing of the test material with saline after absorption does not elute absorbed antibodies, if ice cold saline is employed (3). To 0.1 ml of the last washing of each tube add 0.1 ml of the respective bromelin modified cell suspensions (indicator cells). Centrifuge at 1000 rpm for one minute and ex-

amine macroscopically. If no agglutination is present all free antibodies have been removed.

12. The last washings are discarded and 0.4 cc of saline is introduced to each tube.

13. The tubes are then placed in a water bath (temp. 56°C) and incubated for a period of 15 minutes. The tubes are gently agitated during the incubation period.

14. 0.1 ml of the eluate from each tube is added to 0.1 ml of its respective normal saline indicator cell suspension. Allow to stand for 5 minutes then centrifuge at 1000 rpm for one minute. Read macroscopically. This procedure is repeated testing 0.1 ml of the eluate of each tube against 0.1 ml of its respective bromelin sensitized saline indicator cell suspensions. Read macroscopically.

RESULTS AND COMMENTS

1. Absorbed antisera, which had been eluted, demonstrated a high avidity for bromelin-modified indicator cells as compared with normal indicator cells. In most cases, a 3+ to 4+ agglutination was observed when the eluted sera was tested against the modified cells. This was in contrast to the 1+ or 2+ agglutination obtained with normal indicator cells.

2. Of the 55 stains, only 12, or 21.8%, could be grouped by the Lattes or Crust Method; 44, or 80% by the Absorption Elution method using normal indicator cells; and 51, or 92.7%, using bromelin-modified indicator cells. The increase of almost 13% obtainable results thru the use of bromelin-modified cells reflects the high sensitivity of this enzyme in detecting antibodies. (See Table)

SUMMARY OF TESTS

Known Stains	No.	Lattes or Crust Method	Absorption Normal Cells	Elution Tests Modified Cells
A	15	6	12	14
B	15	4	13	14
AB	10	—	6	9
O	15	2	13	14
Totals.....	55	12	44	51
Percentage.....		21.8%	80%	92.7%

3. Excellent results were obtained in the detection of the absorbed H serum, notably in stains of group O. In testing for the H antigen (on stains of group O), with normal indicator cells, reactions were weak and in some cases non-existent. A

strong 2+ to 3+ agglutination was observed when the same stains were tested utilizing the modified cells and reactions were observed in those stains that gave negative reactions with the normal indicator cells. The H antigen was also detected in some stains of group A, B, and AB with the modified cells.

4. No non-specific or false positive reactions were observed in the control tests or on unstained materials tested. In all aspects, bromelin-modified cells appear to satisfy the rigid requirements of specificity.

5. The use of bromelin eliminates pre-incubation, washings, and other time-consuming and tedious preparations associated with other proteolytic enzymes.

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