Immunochemical Confirmation of Gastrointestinal Bleeding

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ABSTRACT

Hemoglobin was detected by hemagglutination inhibition and by direct slide latex agglutination in extracts from feces from subjects who had gastrointestinal bleeding or who had ingested their own blood. Blood and myoglobin from the meat of other species did not react. A direct slide latex agglutination test for human erythrocyte stroma may give some indication of the site of gastrointestinal bleeding.

Introduction

Gastrointestinal bleeding is a prominent feature of gastrointestinal carcinoma as well as a variety of other conditions. Gross bleeding from a site in the rectum or lower colon with blood clearly visible in the stool specimen or from a higher site resulting in tarry stools gives no problem in detection. Since gross bleeding occurs comparatively late, it is not particularly helpful as an early sign of carcinoma. Methods for detection of occult blood are required. There is some debate in the literature about the usefulness of tests for occult blood in feces for indication of cancer. Some authors state that detection of occult blood in feces is of limited value in early recognition of gastrointestinal cancer,7,12 while others consider such tests of prime importance.9,14,18 Recently, a mass screening for occult blood carried out in a New Jersey county resulted in the discovery of five previously unknown cancers of the colon and rectum.17 Key points of the debate center on the sensitivity of the several chemical tests for peroxidase activity, false positives and false negatives, and the necessity of special dietary preparation. The sensitivity of the chemical methods depends on the particular indicator, the type and concentration of peroxide, type and pH of buffering agents, as well as the preparation of the specimen.1,2 The most sensitive combinations lead to false positives caused by vegetable and bacterial peroxidases and by hemoglobin and myoglobin from meat in the diet. The methods with lesser sensitivity will miss some bleeding. The ideal method would detect small quantities of blood, but not peroxidase of dietary origin. Some have suggested that boiling an extract of a stool specimen will destroy the activity of vegetable peroxidase but not that of hemoglobin.8 It has been the experience of this laboratory that the activity of hemoglobin is destroyed just as rapidly as that of the plant peroxidases.11 The use of the hydroperoxides of the cumene class, which have a greater specificity
for hemoglobin than for the vegetable peroxidases, reduces the false positives from the vegetable sources. There still remains the problem of “false positives” from hemoglobin and myoglobin in a meat diet. These can only be eliminated by a meat free diet or by accepting a test with inadequate sensitivity.

It was shown by this laboratory that antiserum to human hemoglobin did not cross react with hemoglobin of other species with the exception of monkey nor with myoglobin of any species. Immunochemical methods then may be used to differentiate hemoglobin of endogenous origin from that of exogenous origin.

The stroma of erythrocytes is also antigenic. It seems reasonable that some of the antigenic structure could persist in the stool, particularly if the source of bleeding is in the rectum or colon. The use of immunochemical tests for both stroma and hemoglobin may be of help in defining the site of bleeding.

Materials and Methods

**Principle**

The hemoglobin antigen is prepared from human blood and used to immunize rabbits or goats as previously described.

Erythrocyte stroma antigen is isolated from human erythrocytes and used to immunize rabbits.

Hemoglobin is conjugated to formalinized sheep erythrocytes by a one-stage diazobenzidine method or a two-stage pyrrole azide-diazobenzidine method. The conjugate is agglutinated by an antiserum to hemoglobin and the agglutination is inhibited by free hemoglobin. These systems give a hemagglutination inhibition test for human hemoglobin.

Gamma globulin is isolated from antiserum to hemoglobin and absorbed on latex particles. In the presence of antigens from the erythrocyte surface a visible agglutination takes place. This system gives a direct slide test for human hemoglobin.

Gamma globulin is isolated from antiserum to erythrocyte stroma and absorbed on latex particles. In the presence of antigens from the erythrocyte surface a visible agglutination takes place. This system gives a direct slide test for human erythrocyte stroma.

The feces specimen is extracted with a pH 9.2 buffer. The filtered extract is used for the hemagglutination inhibition assay, the direct slide agglutination test for hemoglobin, and the direct slide agglutination test for erythrocyte stroma.

**Antigen Preparation**

**Hemoglobin.** The hemoglobin antigen is prepared from freshly drawn oxalated whole human blood and purified by acrylamide gel electrophoresis as previously described.

**Erythrocyte Stroma.** Human type O blood is collected with oxalate as the anticoagulant. The erythrocytes are centrifuged out and the plasma removed. The cells are washed several times with an equal volume of saline. The cells are lysed with 8 to 10 volumes of distilled water. The lysate is centrifuged at 16,000 rpm for 30 minutes. The hemoglobin solution is decanted and the precipitate is washed twice with water, twice with saline, twice with the pH 9.2 electrophoresis buffer, twice with a pH 4.4, 0.4 M phosphate buffer in 2 M sodium chloride, twice with water and twice with saline. Each time the stroma is centrifuged off at 16,000 rpm for 30 minutes. The stroma is suspended in water, frozen, and lyophilized.

**Antisera Immunization and Bleeding**

**Hemoglobin.** Rabbits are immunized and blood collected as described. Goats can also be used for production of antisera to
immunochemical confirmation of gastrointestinal bleeding

Hemoglobin. The initial injection is made into each of the dew claws of the goat. The amount of antigen used is the same as that for the rabbit. The goat is bled from the jugular. A final bleeding is by carotid cannulation. For both the rabbits and goats, the booster injections may be given subcutaneously as well as intravenously. For subcutaneous boosters, the antigen is mixed with an equal volume of Freund's incomplete adjuvant and homogenized. The antigen is injected into a site in each of the four quarters of the animal.

Stroma. Rabbits are immunized with erythrocyte stroma using the same schedule as for hemoglobin. For the initial foot pad injections, 2.2 mg of the stroma preparation are suspended in 0.8 ml water and mixed with an equal volume of Freund's complete adjuvant. An injection of 0.4 ml is given in each foot pad. For the boosters, each rabbit is given 0.5 ml of a saline suspension containing 1.4 mg stroma per ml.

Antiserum Processing

The rabbit or goat blood is allowed to stand at room temperature for one to two hours to clot. The clot is loosened from the sides of the tube and the tube is refrigerated overnight. The clot is centrifuged at 2,000 rpm for 15 minutes. The serum is poured off and centrifuged again. Complement is destroyed by heating the serum in a water bath at 56° for 30 minutes. Heterophile antibody is removed by absorption with lyophilized formalinized sheep red cells (10 mg per ml sera). The serum is stored undiluted at 60°.

Antisera may be tested for titer and specificity with immunodiffusion and immunoelectrophoresis as described in our earlier papers.

Hemagglutination Inhibition

One-Stage Diazobenzidine Conjugate. A conjugate of hemoglobin and formalinized sheep erythrocytes is prepared by a bis-diazobenzidine coupling procedure described in earlier publications.

Two-Stage Pyrrole Azide-Diazobenzidine Conjugate. The procedure for the preparation of a myoglobin conjugate from the earlier work is used for the hemoglobin conjugate. This is a modification of the Howard and Wild technique for coupling two proteins. In the first step the protein reacts with pyrrole-2-carboxylic acid azide at pH 9 to 10. Next, formalinized sheep red cells are diazotized with bis-diazobenzidine at pH 3 to 4. Finally, the two solutions react at pH 7.

Step 1: 1.08 mg hemoglobin in 2.5 ml saline is mixed with 5 ml phosphate buffer, 0.15 M, pH 7.4, with 6.25 mg sodium bicarbonate. The pH is adjusted to 9.5. Precisely 6.25 mg pyrrole-2-carboxylic acid azide are dissolved in 0.25 ml dioxane and 0.25 ml distilled water. The pyrrole azide is added dropwise to the buffered hemoglobin solution. The pH is maintained at 9.5 with 0.1 N sodium hydroxide. The hemoglobin pyrrole solution is dialyzed for three days against cold phosphate buffer, pH 7.

Step 2: Seven ml of 10 percent formalinized sheep red cells are centrifuged and resuspended in 8 ml sodium acetate buffer, pH 3.5. Two ml bis-diazobenzidine are added to 1 ml of 3.5 acetate buffer and 1 ml of distilled water. This is added dropwise to the red cells and shaken, the pH is adjusted to 3.5 with 0.1 N sodium hydroxide and the mixture is rotated for 30 minutes at room temperature. After this reaction time, the cells are washed four times with sodium acetate buffer and suspended in 8 ml saline.

Step 3: The hemoglobin is added dropwise to the cells and mixed. The pH rises gradually and is finally adjusted to 6.5. The conjugates are rotated at room temperature for four hours. Fifty mg β-naphthol in 4 ml ethyl alcohol is added and mixed with the
conjugate. The conjugate is refrigerated for two days, washed four times with saline and finally suspended in 40 ml saline containing 40 mg sodium azide. The conjugate should be stored for one week before use.

**Latex-γ Globulin Conjugates**

*Preparation of γ globulin*

**Reagents:** 0.4 percent rivanol (400 mg 2-Ethoxy-6,9-diaminoacridine lactate which is dissolved in 100 ml water) and Charcoal-Norit A.

**Procedure:** One ml antiserum to hemoglobin is mixed with 3.5 ml of the rivanol solution. The mixture is centrifuged and the supernatant is decanted. The supernatant is treated with a small amount of charcoal. The charcoal is centrifuged off and the treatment repeated until the supernatant is colorless. (Alternatively, the rivanol may be removed by passing the supernatant through a Sephadex G-25 column.) The supernatant is frozen and lyophilized.

*Preparation of Hemoglobin Slide Conjugate*

**Reagents:** γ globulin from antiserum to hemoglobin and Polystyrene Latex Particles (Dow) 0.234 micron.

**Procedure:** To 1 ml of the latex suspension is added 0.1 ml of an isotonic saline solution of γ globulin (1 mg per ml). This is mixed for 30 minutes with end-over-end tumbbling. The mixture is diluted with an equal volume of saline.

*Preparation of Stroma Slide Conjugate*

**Reagents:** γ globulin from antiserum to stroma. This is prepared from the antiserum to stroma in the same manner as the hemoglobin slide conjugate. Polystyrene latex particles* 0.234 micron.

**Procedure:** To 0.5 ml of the latex suspension is added 0.1 ml of an isotonic saline solution of γ globulin (1 mg per ml). This is mixed for 30 minutes with end-over-end tumbbling. The mixture is diluted with an equal volume of saline.

**Standard Solutions**

**Hemoglobin**

Standard solutions of hemoglobin can be prepared from the antigen solution used for electrophoresis or from whole blood. The standards can be used for testing the performance of the conjugates and the antisera. Usually saline solutions containing 48, 24, and 12 micrograms hemoglobin per milliliter are prepared. The direct correlation of the reactions of the standards with amount of gastrointestinal bleeding is not possible as will be discussed later. Dilutions of blood in saline can be used to verify the performance of the stroma conjugate.

**Bicine Buffer (0.2 m)**

Exactly 3.26 g bicine† per 100 ml are adjusted to pH 8.5 with 5N sodium hydroxide.

**Rabbit Serum**

Non-immune serum is heated and absorbed in the same manner as the antisera.

**Procedure**

**Extraction of Specimen**

Approximately one g of feces specimen is blended with 100 ml of the tris-borate electrophoresis buffer* in a Waring blender for 10 minutes. The mixture is filtered through a rapid filtration paper. The filtrate is tested with Hemastix®. The presence of blood, hemoglobin or myoglobin of human or other species origin is indicated by a large or moderate color with the specimen extract or a dilution of the extract. The immunochemical tests are used for confirming the gastro-intestinal bleeding.

**Hemagglutination Inhibition**

Antiserum to hemoglobin is diluted serially 1:20, 1:40, 1:80, 1:160, 1:320, 1:640,

* Dow.

† Nutritional Biochemicals.
IMMUNOCHEMICAL CONFIRMATION OF GASTROINTESTINAL BLEEDING

TABLE I
Reactions of Extracts

<table>
<thead>
<tr>
<th>Regime</th>
<th>Hemastix™ Wells of Inhibition</th>
<th>Direct Slide Agglutination</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hemagglutination inhibition</td>
<td>Hemoglobin</td>
</tr>
<tr>
<td>Control</td>
<td>Negative</td>
<td>0</td>
</tr>
<tr>
<td>Ingestion</td>
<td>Small</td>
<td>MODERATE</td>
</tr>
<tr>
<td>of 10 ml</td>
<td>Moderate</td>
<td>3</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td>3</td>
</tr>
<tr>
<td>Ingestion</td>
<td>Large</td>
<td>11</td>
</tr>
<tr>
<td>of 20 ml</td>
<td>Moderate</td>
<td>9</td>
</tr>
<tr>
<td>Blood</td>
<td></td>
<td>9</td>
</tr>
<tr>
<td>Ingestion</td>
<td>Large</td>
<td>2</td>
</tr>
<tr>
<td>of 20 ml</td>
<td>Large</td>
<td>2</td>
</tr>
<tr>
<td>Blood</td>
<td>Small</td>
<td>2</td>
</tr>
<tr>
<td>(capsule)</td>
<td>Large</td>
<td>1</td>
</tr>
<tr>
<td>Meat diet</td>
<td></td>
<td>0</td>
</tr>
</tbody>
</table>

latex-globulin suspension from the antiserum to stroma.

Results

As shown in table I, both the direct slide agglutination test and the hemagglutination inhibition test were positive following the ingestion of 10 or 20 ml whole human blood either as liquid or a lyophilized blood in gelatin capsules. About 10 hours after ingestion, the first positive test was obtained and stool specimens may be positive for several days. Extracts from this amount of ingested blood did not give a precipitin line against anti-hemoglobin on immunodiffusion. The stroma test was negative following the ingestion of blood by either means. This ingestion corresponds to bleeding at the level of the stomach, or above, or the duodenum. A specimen from a subject with bleeding in the lower part of the colon gave a strong positive test for stroma. The exact level at which the stroma test becomes positive is not yet known.

Neither of the tests for human hemoglobin nor the test for stroma was positive with extracts of stools from subjects on a meat diet, although the peroxidase test was strongly positive.
TABLE II
LABORATORY SPECIMENS

<table>
<thead>
<tr>
<th>Specimen</th>
<th>Hemastix®</th>
<th>Hemagglutination Inhibition</th>
<th>Direct Slide Agglutination Wells of Inhibition</th>
<th>Hb</th>
<th>Stroma</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Small</td>
<td>0</td>
<td>1+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2 Small</td>
<td>4</td>
<td>3+</td>
<td>2+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>3 Large</td>
<td>8</td>
<td>4+</td>
<td>3+</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>4 Moderate</td>
<td>9</td>
<td>3+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5 Moderate</td>
<td>7</td>
<td>4+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>6 Large</td>
<td>9</td>
<td>3+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>7 Moderate</td>
<td>3</td>
<td>2+</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>8 Large</td>
<td>6</td>
<td>3+</td>
<td>4+</td>
<td>2+</td>
<td>2+</td>
</tr>
<tr>
<td>9 Large</td>
<td>11</td>
<td>3+</td>
<td>4+</td>
<td>2+</td>
<td>2+</td>
</tr>
</tbody>
</table>

In table II are shown results obtained with specimens submitted to a local laboratory. Specimen 6 reacted for hemoglobin but not stroma, indicating bleeding from the duodenum or above, while specimens 8 and 9 with bleeding from the colon were positive for both hemoglobin and stroma.

Discussion

Sensitivity

The exact sensitivity of the immunochmical tests is difficult to define. In aqueous solutions the hemagglutination inhibition is capable of detecting 2 µg per ml and the slide test is positive at 4 to 8 µg per ml. This can not be compared to the stool extracts since the extracts contain the hemoglobin in various stages of degradation all the way down to hematoporphrin. It is more informative to say that the tests will be positive following the ingestion of 10 ml of blood.

The state of degradation of hemoglobin on passing through the gastrointestinal tract probably explains variable results obtained with different means of detecting it immunochmically. Hemoglobin could not be detected by immunodiffusion of the extracts from the blood ingestion experiments. It seems logical that immunodiffusion may require an intact structure, hemagglutination inhibition may require somewhat less of the original structure and direct slide agglutination may be positive with fragments of the original globin. The degradation undoubtedly continues after the specimen is collected; thus, testing should be carried out as soon as possible.

In the colon, the hemoglobin is probably in an anaerobic environment, after collection of the specimen, it may be in an aerobic environment resulting in some shifts of the degradation.

The two-stage conjugate gave a much clearer titration than the one-stage conjugate but the two-stage conjugate required a greater amount of antiserum. This probably means a much smaller number of antigen-antibody sites are involved in the case of the two-stage conjugate.

Normal Levels

There is disagreement about the level of blood in the stool of healthy people. Based on peroxidase activity, from 0.3 to 1.5 ml per day has been reported. With Cr$^{51}$ tagging, Cameron reported 0.3 to 1.3 ml per day, but this may be an over estimation since some of the Cr is excreted in the bile.

Since extracts from stools from subjects on a meat diet did not react immunochmically, preliminary dietary restrictions are not necessary.

Clinical Résumé

It is important to point out that although these tests indicate bleeding somewhere in the gastrointestinal tract, it does not necessarily indicate that carcinoma is present. Bleeding can be from ruptured varicose veins of the esophagus or stomach, swallowed blood from pulmonary hemorrhage, carcinoma of the esophagus, stomach or intestine, ulcer of the stomach or duodenum, intestinal ulcers owing to typhoid fever or tuberculosis, embolism of the superior mesenteric artery, venous thrombo-
sis of mesenteric veins, ulcerative colitis, bacillary and amebic dysentery, volvulus, intussusception, enteritis, hemophilia, hemolytic jaundice and/or hemorrhoids. In spite of these other causes of blood in the feces, detection of occult blood in the stool is a significant indicator of carcinoma. Blood in the stool has been reported for 80 percent of patients with gastrointestinal cancer. Hastings believes that tests for occult blood would be more effective in increasing the incidence of early detection of gastrointestinal cancer than appealing to subjects to have a proctosigmoidoscopic examination.

References


