Some Interrelationships of Blood and the Fava Bean Principle in Vitro

By William P. Creger, M.D. and Houghton Gifford, M.D.

Little is known of the mechanism of red cell destruction due to the Fava bean, though many thousands of cases occur in Italy each year, and over a hundred papers had appeared on the subject by 1941. A small number of cases of hemolytic anemia due to Vicia fava have been reported in this country, but there is some reason to think the disease is actually more frequent than the literature would indicate. Not only is the bean grown extensively here, prepared in fresh-frozen form and imported canned, but there is a large indigenous Italian population eating the bean with some regularity.

In a review of the Italian literature, written in English, Luisada presented clinical descriptions of the disease and discussed the various pathogeneses suggested. He favored an "allergic theory" based largely on the clinical features of the disease, as well as on experiments involving sensitization of rabbits, and on certain skin tests. He did not mention any studies of in vitro relationships of the Fava bean to human red cells. Leeks studied the effects of a "concentrated extract" of Vicia fava on human red cells, but was unable to demonstrate agglutination or hemolysis. Boyd, in his search for type-specific hemagglutinins in 262 kinds of beans, mentioned that a 1:3 saline extract of Fava bean agglutinated all types of human erythrocytes, but he was not concerned more specifically with this particular legume.

Many beans have been noted to possess hemagglutinative properties, and several have been used to produce hemolytic anemia experimentally. Concanavalin A, a crystalline substance prepared from the jack bean, genus Canavalia, has been most studied in this regard. In vitro, Concanavalin A has been found to be agglutinative for many animal cells, though not for human cells. In vivo, this substance has produced a hemolytic anemia, though the precise mode of action was uncertain.

The purpose of this paper is to report on certain relationships between the Fava bean principle and human red cells, when studied in saline, serum and in various colloidal diluents.

Materials

1. Dried Fava* beans were ground in an ordinary meat grinder to a coarse powder and placed in a Waring Blender with four parts of normal saline. After spinning for two minutes, the mixture was centrifuged one-half hour at 2000 r.p.m., and the supernatant liquid filtered through paper; merthiolate was added to a concentration of 1:25,000. The extract was kept at a temperature of 5°C, and cultures were occasionally made to ensure using a sterile preparation. Dilutions of this 1:5 normal saline extract of dried Fava bean consti-
tuted the agent to be tested. Three additional varieties of bean extracts, in 1:5 normal saline diluent, were prepared, i.e., fresh Fava bean, the Common Vetch (the closest botanical relative of the Fava bean) and the Lima bean. These three bean extracts were tested with red cells as given below.

2. Freshly drawn human and animal red cells were washed three times in 3 cc. portions of normal saline, and made up to a 1 per cent suspension in normal saline. They were used only on the day of drawing.

3. A number of colloidal diluents were used in studying the effects of saline extracts of beans on red cells. These were: Armour's 30 per cent bovine albumin, undiluted serum of group AB, 1:6 LePage's glue in normal saline and 10 per cent normal saline solutions of certain gums—gum acacia, gum tragacanth and gum Ghatti. The gums were prepared as follows: 10 Gm. of the gum were mixed with 90 cc. of normal saline, the pH adjusted to neutrality, and the mixture autoclaved at ten pounds' pressure for ten minutes. These preparations were kept at 5 C.

<table>
<thead>
<tr>
<th>Serial Dilutions of Bean Extract</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
<th>320</th>
<th>640</th>
<th>1280</th>
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<tbody>
<tr>
<td>1 Fava Bean Extract plus human red cells</td>
<td>++</td>
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</tr>
<tr>
<td>2 Fava Bean Extract plus rabbit red cells</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>--</td>
</tr>
<tr>
<td>3 Common Vetch Extract plus human red cells of group A</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
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</tr>
<tr>
<td>4 Lima Bean Extract plus human red cells of group A</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>+++</td>
<td>++</td>
<td>--</td>
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</tr>
<tr>
<td>5 Fava Bean Extract plus serum, incubate, then add cells</td>
<td>±</td>
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</table>

EXPERIMENTS AND RESULTS

1. The Effect of Saline Extracts of Certain Beans on Red Cells in Normal Saline

One drop of a 1 per cent suspension of thrice-washed human or rabbit red cells was placed with 0.2 cc. dilutions of 1:5 normal saline extract of dried Fava beans; the tubes were placed in the 37 C. water bath for one-half hour, then spun for 1 to 2 minutes at 2000 r.p.m. in an angle head centrifuge and examined for agglutination.

The intensity of hemagglutination by Fava bean extract is recorded in lines 1 and 2 of table 1. All clumps were macroscopically visible, though all readings reported were made with the aid of a dissecting microscope. The agglutination was very intense and of the order seen in red cell typing. Tubes of 1:10 and 1:20 dilution exhibited a solid button of agglutinated cells which, on rolling the tube, floated free without rupturing. Guinea pig and albino rat red cells were affected in a manner similar to human and rabbit erythrocytes. Human red cells were clumped by the Fava bean extract, apparently without regard to blood groups. Fresh bean extract was entirely similar in hemagglutinative activity to dried bean extract, though slightly weaker; with the same dilution technic as shown above for human cells, clumping was noted in a dilution of 1:40, as contrasted
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with 1:80 for the dried bean extract. If red cells, previously treated with Fava bean extract and centrifuged for reading, were allowed to stand for one-half hour at 25 C., it was noted that the last few positive dilutions became equivocal in result. The previous level and strength of agglutination could be restored by recentrifugation.

Serial dilutions of 1:5 saline extract of Common Vetch were made, and a drop of a 1 per cent suspension of thrice-washed Rh negative human red cells of group A was added to each tube. Incubation, centrifugation and reading technic were as performed with the Fava bean extract.

As indicated in line 3 of table 1, extract of Common Vetch produced agglutination of red cells in higher titer than did extract of Fava bean.

After standing at 25 C. for one-half hour, agglutination in all tubes except that of 1:10 dilution disappeared; recentrifugation produced the strong, high titer again.

Serial dilutions of 1:5 normal saline extract of Lima bean were also made and human red cells of group A used in determining the titer of activity. The results contained in line 4, table 1, show that extract of Lima bean was considerably weaker in hemagglutinative power than was extract of Fava bean. The behavior of these reactions on standing and recentrifugation was the same as noted with the Vetch extract.

The agglutinative effect of 1:10 dilution of 1:5 normal saline extract of dried Fava bean on human red cells in saline diluent, was removed with three washings of 37 C. normal saline, and almost completely removed with 25 C. saline. On the other hand, six washings with normal saline at a temperature of 5 C. failed to do more than weaken the agglutination slightly. Similar effects were noted on washing erythrocytes agglutinated with Lima bean and Common Vetch extracts. However, as shown below, this loss of activity in saline following saline washings did not mean that the hemagglutinin or its effect had been completely removed from the cell.

2. The Effect of Fresh Human Serum on the Hemagglutination of Human Red Cells by Fava Bean Extract

Two-tenths cc. volumes of normal saline extract of dried Fava bean and freshly drawn human serum were incubated together for one-half hour at 37 C. and serial dilutions made of this mixture; red cells were added and the test made as usual. The results indicated a striking inhibition of agglutination attributable to the action of the serum. (Compare lines 1 and 5 in table 1.)

Similar results were obtained in tests with rabbit cells and rabbit serum. For example, rabbit red cells plus Fava bean extract yielded clumps through a dilution of 1:640 of the extract; but if the extract was first exposed to an equal volume of rabbit serum and then the red cells added, clumps were found at a dilution of 1:5 but not at a dilution of 1:10. It was also found that even if Fava bean extract and cells were incubated for thirty minutes first, and then serum added, the hemagglutination inhibition action of serum was still evident, i.e., titers fell from the control value of 1:640 to 1:10. The hemagglutinative inhibitory action of serum was also evident in the cases of the Lima bean extract and the common Vetch extract.
No hemolysis was observed on the addition of fresh human serum to Fava-sensitized red cells. There was no evidence that complement-dependent hemolysis occurred when Fava-sensitized red cells were placed in human serum. It was also noted that two human sera with moderately high levels of anti-A hemolysin were no more hemolytic for Fava-sensitized cells of group A than for cells of group A alone. Nor did red cells sensitized to Fava bean extract exhibit increased fragility, either to hypotonic saline or to mechanical agitation.

3. The Effects of Various Colloidal Diluents on the Hemagglutinative Activity of Certain Bean Extracts for Human Red Cells

A. One drop of a 1 per cent suspension of thrice-washed human red cells was placed with 0.2 cc. dilutions of the 1:5 normal saline extract of dried Fava bean as above, and incubated for one-half hour at 37 C.; two drops of 10 per cent acacia (prepared as indicated above) were then added to each tube, and incubation followed for another one-half hour before centrifugation and reading.

As indicated in table 2, acacia* potentiated the hemagglutinative effect of saline Fava bean extract 100-fold. (Compare lines 1 in tables 1 and 2.)

B. One drop of the red cell suspension was exposed to each of the following dilutions of Fava bean extract for one-half hour at 37 C.: 10, 100, 1,000, 10,000, 100,000. The cells were then washed twice in normal saline at 25 C. and inspected under the dissecting microscope. If no agglutination was present in the saline, the tubes were decanted, and two drops of 10 per cent acacia added to each tube. After one-half hour incubation the tubes were centrifuged and read. Control

* In all acacia experiments, the red cells must be freshly drawn, as acacia sometimes clumps unsensitized red cells which have been kept overnight. A suitable control, consisting of unsensitized cells plus acacia, or other diluent used, accompanied each analogous experiment with sensitized cells, and did not exhibit agglutination on centrifugation.

Table 2.—Effects of Colloids on Bean Extract Agglutinations of Human Red Cells

<table>
<thead>
<tr>
<th>Serial Dilutions of Bean Extract</th>
<th>10</th>
<th>100</th>
<th>1000</th>
<th>10,000</th>
<th>100,000</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Fava Bean Extract plus red cells, incubate, then add acacia...</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>-</td>
</tr>
<tr>
<td>2 Fava Bean Extract plus red cells, incubate, wash, then add acacia</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
<td>-</td>
</tr>
<tr>
<td>3 Fava Bean Extract plus red cells, incubate, wash, then add albumin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4 Fava Bean Extract plus red cells, incubate, wash, then add serum of group AB</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>5 Fava Bean Extract plus red cells, incubate, wash, then add 1:6 glue*</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>6 Lima Bean Extract plus red cells, incubate, wash, then add acacia</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>7 Common Vetch Extract plus red cells, incubate, wash, then add acacia</td>
<td>++++</td>
<td>++++</td>
<td>++++</td>
<td>++</td>
<td>-</td>
</tr>
</tbody>
</table>

* Five parts normal saline, plus one part LePage's glue.
tubes were: unsensitized cells and acacia, and sensitized cells plus two drops of 30 per cent bovine albumin, 1:6 saline-diluted LePage's glue, and serum of group AB. The results of this experiment are presented in lines 2 through 5 of table 2. In sharp contrast to the action of acacia, three other colloidal diluents did not potentiate the hemagglutinative effect of Fava bean extract.

C. Rh negative human red cells of group A were sensitized to the following dilutions of 1:5 normal saline extract of Lima bean and Common Vetch: 10, 100, 1,000, 10,000, 100,000. The sensitized cells were washed in normal saline at 25 C. until no agglutination was seen on centrifugation (usually 2 to 3 washings). Two drops of 10 per cent acacia were added to each tube, the tubes incubated for one-half hour at 37 C., and centrifuged and read.

As shown in lines 6 and 7 of table 2, acacia potentiated the hemagglutinative effects of Lima bean and Common Vetch extracts in a manner similar to its effects with Fava bean extract. (Compare line 3, table 1, and line 7, table 2; also line 4, table 1, and line 6, table 2.)

Ten per cent normal saline preparations of gum Ghatti and gum tragacanth did not potentiate clumping of similarly treated erythrocytes, while bovine albumin weakly imitated the action of acacia in the above scheme with Vetch and Lima bean extracts. We have not observed such an imitation by albumin with any Fava bean extract.

In agreement with Boyd's findings12 the Lima bean extract did not agglutinate human red cells of groups B and O in saline diluent. However, using the acacia method, it was evident that Lima bean extract had an agglutinative action on all types of human red cells; this activity was not evident in saline with cells of groups B and O, and was only weakly present with cells of group A. When cells of groups B and O were treated with 1:100 dilutions of 1:5 saline extract of Lima bean, there were no reactions in saline, but after washing the cells and adding acacia moderately strong clumps were observed.

4. The Demonstration with Acacia of the Transfer of Fava Principle from Cell to Cell

One-tenth cc. (packed) red cells were placed with 0.5 cc. of 1:100 dilution of 1:5 normal saline extract of dried Fava bean for one-half hour at 37 C. The cells were then washed twice in normal saline at 25 C., 0.5 cc. of normal saline added, and then placed in the water bath at 56 C. for ten minutes. The eluate was recovered and placed over one drop of unsensitized red cell* suspension for one-half hour at 37 C.; the cells were then washed twice with normal saline at 25 C. and two drops of 10 per cent acacia added. The tube was then incubated for one-half hour at 37 C., centrifuged and read. Controls consisted of (a) unsensitized cells* and acacia, (b) unsensitized cells* eluted at 56 C. for ten minutes, the eluate placed with one drop of unsensitized cell* suspension as above, incubated, washed and acacia added.

The results shown in table 3 indicate that the hemagglutinative principle of the Fava bean extract can be transferred from cell to cell.

An identical eluate transfer of bean principle with positive acacia test was demonstrated using the common Vetch extract; but extract of Lima bean,

* From same donor, same drawing, as the cells which were sensitized and eluted.
evidently hemagglutinatively weaker, could not be shown to transfer from sensitized to unsensitized cells with the technic used.

5. The Nature of the Fava Bean Principle

Only an incomplete attempt has been made thus far to characterize the Fava bean principle.

A. The effect of heat. 1:10, 1:20, 1:40, 1:80 and 1:160 dilutions of normal saline extract of dried Fava bean were subjected to temperatures of 37, 56, 66 and 76 C. for 10 minutes, and one drop of a 1 per cent suspension of human red cells added to each tube. They were incubated at 37 C. for one-half hour, centrifuged and read.

The results, presented in table 4, demonstrated that the agglutinating property of the Fava bean extract was inactivated by subjecting it to a temperature of 76 C. for 10 minutes.

B. The effect of dialysis on the hemagglutinative activity of the Fava bean extract. Two cc. of a 1:5 normal saline extract of dried Fava bean were placed in a small cellophane bag, and the latter placed in 2 cc. of normal saline. Twenty-four hours later there was no agglutinative activity for red cells outside the bag, while the usual activity was present within the bag.

6. The Fraction of Serum Responsible for the Inhibition of Hemagglutination by Normal Saline Extract of Dried Fava Bean

Certain serum protein fractions were tested for a possible role in the inhibition of hemagglutination by the Fava bean extracts. These fractions were: human albumin alone, albumin plus alpha and beta globulins, albumin plus alpha globulin alone and a concentrated gamma globulin solution.*

* The serum fractions were kindly prepared for us by Dr. H. Loeb of the Department of Pediatrics, Stanford University School of Medicine, by a fractional technic with sodium sulfate precipitation. The concentrated gamma globulin solution may be obtained from Squibb and Co.
Equal volumes (0.2 cc.) of the above serum fractions and 1:5 normal saline extract of dried Fava bean were incubated for one-half hour at 37 C., and a drop of 1 per cent suspension of human red cells added. The tubes were incubated at 37 C. for one-half hour, centrifuged and read.

The results indicated that human serum albumin and alpha globulin were completely without power to inhibit the agglutination of red cells by the bean extract. Beta globulin was inferred to have perhaps slight activity in this regard, but the gamma globulin preparation was shown to have had the principal role in inhibiting agglutination of red cells by the Fava bean extract, as shown in line 5 of table 1.

**Discussion**

Normal saline extracts of the Fava bean exhibited powerful agglutinative effects on all human red cells regardless of blood group, and on the red cells of at least three unrelated animal species. The agglutination was greatly potentiated by 10 per cent acacia, but by no other colloidal diluent or gum tried. Serum was capable of inhibiting the hemagglutination, and the active fraction of serum in this regard appeared to be gamma globulin. The hemagglutinating power of Fava bean extract was not due to a physicochemical alteration in the cell membrane in the manner of acid hemagglutination. Rather, there appeared to be an absorptive effect in which a discrete chemical fraction of the Fava bean extract adhered to the red cell surface and could be transferred from cell to cell, as demonstrated by the elution technic used.

What, if any, is the relationship of the above in vitro activity to in vivo red cell destruction following contact with the bean or its plant’s pollen, is not clear. Fava-sensitized cells in the test tube were not unusually fragile nor abnormally subject to the action of complement or hemolysin, and no mechanism has been suggested for the final mode of hemolysis in vivo by any of the findings in vitro. The general obscurity in regard to the ultimate mechanism of in vivo red cell destruction in many hemolytic processes is well reviewed by Ham and Castle, and by Dameshek and Miller.

In any consideration of the hemolytic process of Favism, certain descriptive elements must be considered as peculiar to this disease. Thus, the Italian authors stress the familial incidence of the disorder; the repeated attacks in one person, while others never suffer the clinical disease despite much contact with the bean; the “allergic nature” of some of the manifestations of the disease, and contact with minute amounts of the agent producing in some persons severe symptoms and anemia. There appears to be a very marked individual susceptibility to the action of the Fava bean, which is out of all proportion to the degree of contact with the active agent. The elucidation of the in vitro action of the Fava bean principle has been the chief object of this study. Further experiments concerning the antigenicity of the bean and its chemical fractions in animals and in man in vivo, are under way and will be reported subsequently.

It seems possible that the potentiating by acacia of the agglutination of the Fava-sensitized cell might form the basis of a diagnostic test for Favism in its acute stages. As yet we have been unable to apply this test because of the rarity of the disease in the San Francisco area.
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Summary

1. Saline suspensions of human red cells, as well as those of several animal species, were agglutinated by normal saline extracts of the Fava bean.
2. This agglutination was potentiated in titer 100-fold in a medium of 10 percent acacia, as a diluent.
3. The inhibition of the hemagglutination action of the Fava bean extract by human serum was apparently attributable to the gamma globulin fraction.
4. The Fava bean principle could be transferred from cell to cell, as shown by heat-elution and acacia technics.
5. Fava-sensitized red cells did not exhibit increased susceptibility in the test tube to complement, hemolysin, or osmotic or mechanical fragility.
6. The mechanism of in vivo red cell destruction in Favism is as yet unknown, but a special immunologic susceptibility to the action of the bean's principle is suspected in certain persons.
7. It is suggested that the relation of acacia to Fava-sensitized red cells may form the basis of a diagnostic test for Favism in the early, acute stages of the disease.

REFERENCES

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