

# The effect of the sequence of erythrocyte sensitization on the bis-diazotized benzidine hemagglutination reaction

Phil Gold, M.D., Ph.D.,\* Jaqueline Jonson, R.T., and  
Samuel O. Freedman, M.D., Montreal, Quebec, Canada

*The BDB-hemagglutinating properties of six different antigen-antibody systems were studied. In each case two tests were performed in which the erythrocytes were sensitized by the addition of BDB either before or after the addition of the antigen. It was found that in those systems where the antigenic determinants were protein in nature the two methods of sensitization gave rise to virtually identical titers. However, in the systems in which the antigens contained phenolic and/or carbohydrate determinant groups, markedly higher titers were obtained if the BDB was mixed with the erythrocytes before rather than after the addition of the antigenic material. It is suggested that the nonspecific adsorption of carbohydrate and phenolic groups onto red cell surfaces in the absence of BDB is the most likely explanation for this phenomenon. This dual method of BDB hemagglutination has potential applications as a method of investigating the chemical nature of antigenic determinants of unknown composition.*

The use of bis-diazotized benzidine (BDB) to couple protein-containing antigen molecules to erythrocytes during passive hemagglutination reactions was first described by Pressman and associates<sup>1</sup> in 1942. The BDB hemagglutination technique has since served as a sensitive method for the detection of antibodies to a wide variety of antigens.<sup>2-7</sup> The original procedure has, however, undergone a number of alterations. One important change concerns the sequence in which the BDB and the antigen are added to the erythrocyte suspension in the process of passive sensitization of the red cells. Originally the erythrocytes and BDB were mixed *before* the addition of the antigen,<sup>1</sup> whereas the more usual method followed at present is to add the BDB *after* the erythrocytes are mixed with the antigen.<sup>2, 3, 4, 6, 7</sup>

Experiments performed in our laboratory<sup>8</sup> revealed that in the case of a

---

From the McGill University Clinic of the Montreal General Hospital.

Supported by grants from The Medical Research Council of Canada, Ottawa, Ontario, The Department of National Health and Welfare, Ottawa, Ontario, and the National Cancer Institute of Canada, Toronto, Ontario.

Received for publication July 13, 1965.

\*Fellow of the Medical Research Council of Canada.

human colonic tumor-specific antigen-antibody system the hemagglutination titers obtained were markedly dependent upon whether the BDB was added to the red cell suspension before or after the addition of the antigenic material. The present investigation was undertaken to determine whether the hemagglutination reactions of a number of other antigen-antibody systems could be similarly influenced by the manner in which the erythrocytes were treated during the course of passive sensitization.

## MATERIALS AND METHODS

### Antigen-antibody systems

1. *Bovine serum albumin (BSA) system.* Bovine albumin, powdered fraction V (Cohn), was purchased from Brickman & Co., Montreal, Canada. Anti-BSA antiserum was prepared by the immunization of rabbits with the BSA preparation.

2. *Tuberculin system.* Tuberculin ("Corper's"), purified protein derivative (PPD) (Stock Lot No. X-0335), was provided through the courtesy of Parke, Davis & Company, Detroit, Michigan. Sera were obtained from human donors with active tuberculosis, from healthy individuals with positive tuberculin skin reactions, and from healthy individuals with negative tuberculin skin reactions.

3. *Ragweed system.* The antigenic material employed was an aqueous extract of ragweed pollen. Sera were obtained from patients with clinical evidence of ragweed allergy and intradermal skin reactions to ragweed extract, from human subjects showing no evidence of ragweed sensitivity, and from rabbits immunized with ragweed extract.

4. *Chlorogenic acid-human serum albumin (HSA) conjugate system.* A chlorogenic acid-HSA conjugate was prepared by the interaction of chlorogenic acid with HSA in the presence of phenol oxidase.<sup>9</sup> Chlorogenic acid was purchased from the Aldrich Chemical Co., Milwaukee, Wisconsin, and recrystallized three times from water in our laboratory. Sera were obtained from patients allergic to green coffee dust who showed intradermal reactions to chlorogenic acid, nonallergic human donors, and rabbits immunized with either the chlorogenic acid-HSA conjugate or free chlorogenic acid. The rabbit antisera were always absorbed with an excess of HSA prior to testing.

5. *Blood group-specific A,B system.* The solution of blood group-specific A and B substances was purchased from Merck Sharp & Dohme, Montreal, Canada, and the anti-A,B blood grouping serum was obtained from Certified Blood Donor Service, Inc., Jamaica, New York.

6. *Human colonic tumor-specific system.* The pooled human colonic cancer tissue extract and the absorbed rabbit tumor-specific antiserum were prepared as previously described.<sup>8</sup>

### Hemagglutination

The general BDB hemagglutination procedure followed has been previously described.<sup>8</sup> The antigen under consideration was linked to rabbit red cells in all cases except in tests involving the A,B blood group system where human type O cells were employed. In every case two separate tests were per-

formed on the same day in which the erythrocytes were sensitized by the addition of BDB either 1 to 2 minutes before or 1 to 2 minutes after the antigen was mixed with the red cell suspension. Erythrocyte sensitization was always carried out at room temperature and hemagglutination patterns were allowed to develop at 4° C. for approximately 18 hours. The optimal proportions of BDB and antigen required for each test system were established by the use of appropriate controls. These proportions ranged from 0.05 mg. antigen protein and 0.5 ml. BDB in the blood group system to 6.0 mg. antigen protein and 1.0 ml. BDB in the chlorogenic acid-HSA system. In any given system the optimal proportions of BDB and antigen were found to be identical regardless of whether the BDB was added to the erythrocytes before or after the addition of antigen.

The controls employed included one series of tubes in which the antiserum was replaced by phosphate buffer, and another series in which the erythrocytes and antigen were mixed without the addition of BDB. Under optimal conditions these tests were invariably negative. In all cases control studies were performed with the sera of humans or of animals having no history or other evidence of previous exposure to the antigen under consideration (Table I). Finally, repeated checks were made for the presence of isoagglutinins in each test system.

Approximately 18 hours elapsed from the time the cells were sensitized to the time the patterns were read. During this interval no apparent difference in stability was noted between the cells sensitized by the addition of BDB after the antigen and those sensitized in the reverse order.

## RESULTS

From a comparison of hemagglutination titers A and B listed in Table I the six antigen-antibody systems studied would appear to fall into three distinct categories. Tests performed with the BSA and the tuberculin systems showed virtually no variation in titer when the BDB was added to the red cell suspension before rather than after the addition of the antigen in the process of erythrocyte sensitization. On the other hand, studies with the systems involving the chlorogenic acid-HSA conjugate, the A,B blood group substances, and the human colonic tumor-specific antigens yielded either negative or very low values when the erythrocytes were sensitized by adding the BDB *after* the addition of the antigen. However, when the red cells were sensitized by adding the BDB *before* the antigen, high titers were obtained.

Between these two extremes, the tests with the ragweed system revealed that although positive results could be achieved between ragweed and antiragweed when the erythrocytes were sensitized by adding the antigen prior to mixing with BDB, significantly higher titers were obtained when the sequence of BDB and antigen addition was reversed.

## DISCUSSION

The experimental findings indicate that the sequence in which BDB and antigen are added to the erythrocyte suspension in the process of passive sensi-

**Table 1.** Comparison of hemagglutination titers obtained with erythrocytes sensitized by the addition of BDB either before or after the addition of antigen

| Antiserum                                                                                                          |           | Hemagglutination                  |                                    |
|--------------------------------------------------------------------------------------------------------------------|-----------|-----------------------------------|------------------------------------|
|                                                                                                                    |           | Titer A<br>(BDB after<br>antigen) | Titer B<br>(BDB before<br>antigen) |
| <i>Bovine serum albumin (BSA)</i>                                                                                  |           |                                   |                                    |
| Pooled rabbit anti-BSA antiserum                                                                                   |           | 40,960                            | 20,480                             |
| Control serum from nonimmunized rabbit                                                                             |           | 0                                 | 0                                  |
| <i>Tuberculin purified protein derivative (PPD)</i>                                                                |           |                                   |                                    |
| Human subjects with active tuberculosis                                                                            | Patient 1 | 5,120                             | 5,120                              |
|                                                                                                                    | Patient 2 | 40,960                            | 40,960                             |
| Healthy human subjects with positive tu-<br>berculin skin reactions                                                | Patient 1 | 640                               | 640                                |
|                                                                                                                    | Patient 2 | 640                               | 1,280                              |
| Healthy human subject with negative tu-<br>berculin skin reactions                                                 |           | 40                                | 40                                 |
| <i>Water-soluble ragweed extract (WSR)</i>                                                                         |           |                                   |                                    |
| Pooled rabbit antiragweed antisera                                                                                 | Pool 1    | 10,240                            | 163,840                            |
|                                                                                                                    | Pool 2    | 12,800                            | 204,800                            |
|                                                                                                                    | Pool 3    | 40,960                            | 655,360                            |
| Control sera from nonimmunized rabbits<br>(3 individual specimens)                                                 |           | 0                                 | 0                                  |
| Human subjects sensitive to ragweed and<br>receiving hyposensitization therapy                                     | Patient 1 | 1,280                             | 20,480                             |
|                                                                                                                    | Patient 2 | 2,560                             | 20,480                             |
|                                                                                                                    | Patient 3 | 12,800                            | 204,800                            |
| Human subjects sensitive to ragweed but<br>not receiving hyposensitization therapy                                 | Patient 1 | 0                                 | 160                                |
|                                                                                                                    | Patient 2 | 160                               | 2,560                              |
| Control human subject without ragweed<br>sensitivity                                                               |           | 0                                 | 0                                  |
| <i>Chlorogenic acid-human serum albumin<br/>(HSA) conjugate</i>                                                    |           |                                   |                                    |
| Rabbit anti-chlorogenic acid-HSA antisera                                                                          | Rabbit 1  | 200                               | 40,960                             |
|                                                                                                                    | Rabbit 2  | 400                               | 327,680                            |
|                                                                                                                    | Rabbit 3  | 400                               | 655,360                            |
| Rabbit anti-chlorogenic acid antisera                                                                              | Rabbit 1  | 0                                 | 163,840                            |
|                                                                                                                    | Rabbit 2  | 0                                 | 327,680                            |
| Control sera from nonimmunized rabbits<br>(8 individual specimens)                                                 |           | 0                                 | 0                                  |
| Human subjects sensitive to green coffee<br>bean and receiving hyposensitization ther-<br>apy to chlorogenic acid  | Patient 1 | 0                                 | 160                                |
|                                                                                                                    | Patient 2 | 0                                 | 160                                |
|                                                                                                                    | Patient 3 | 0                                 | 320                                |
|                                                                                                                    | Patient 4 | 0                                 | 320                                |
|                                                                                                                    | Patient 5 | 0                                 | 320                                |
|                                                                                                                    | Patient 6 | 0                                 | 320                                |
|                                                                                                                    | Patient 7 | 0                                 | 640                                |
|                                                                                                                    | Patient 8 | 0                                 | 640                                |
| Human subject sensitive to green coffee<br>bean but not receiving hyposensitization<br>therapy to chlorogenic acid |           | 0                                 | 320                                |
| Control human subjects without green coffee<br>bean sensitivity (8 individual specimens)                           |           | 0                                 | 0                                  |
| <i>Blood group-specific A, B substances</i>                                                                        |           |                                   |                                    |
| Pooled human anti-A, B antiserum<br>(Group O donors)                                                               |           | 0                                 | 15,360                             |
| Human serum from group AB donors<br>(3 individual specimens)                                                       |           | 0                                 | 0                                  |
| <i>Human colonic tumor extract</i>                                                                                 |           |                                   |                                    |
| Pooled rabbit colonic tumor-specific anti-<br>serum                                                                |           | 0                                 | 204,800                            |
| Control sera from nonimmunized rabbits<br>(6 individual specimens)                                                 |           | 0                                 | 0                                  |

tization of red cells markedly influences the hemagglutination titers in some antigen-antibody systems but has no effect in others. Since the BDB and erythrocytes may be considered to be relatively uniform factors in tests performed with any given system, the explanation for the variability between systems must be found in the properties or composition of the antigens themselves.

The hemagglutination titers obtained with the BSA and tuberculin systems were independent of the sequence of BDB and antigen admixture with the red cells. The BSA prepared from Cohn's fraction V consists of about 98 per cent albumin and 2 per cent  $\alpha$ -globulin,<sup>10</sup> and thus represents a relatively pure, and almost totally protein, antigenic material. The tuberculin PPD employed contains a number of constituents including proteins, nucleoproteins, polysaccharides, and lipids. However, experiments recently performed in our laboratory indicate that antibodies against tuberculin PPD detected by the BDB-hemagglutination procedure are probably directed primarily against one or more protein groups. Similar conclusions have been reported by Cole and Farrell.<sup>4</sup> Thus, the antigenic determinants of the systems which were not influenced by the manner of red cell sensitization were protein in nature.

In those systems where higher hemagglutination titers were observed when the BDB was added to the erythrocytes before rather than after the addition of the antigen, the determinant groups consisted of nonprotein constituents. The studies between the chlorogenic acid-HSA conjugate and homologous rabbit antisera were all performed after the antisera had been absorbed with an excess of HSA. Furthermore, reactivity was observed between the conjugate and the sera of animals immunized with free chlorogenic acid alone. The antigenic determinant group in this system would, therefore, appear to be related to the chlorogenic acid component. A similar situation would seem to hold true in tests with the human antisera, since positive results were obtained only with the sera of patients who were skin sensitive to chlorogenic acid. The chlorogenic acid molecule (3-caffeoylquinic acid) is a phenolic acid esterified to a carbohydrate.

It is well established that the blood group-specific A and B substances are mucopolysaccharides consisting of linked polypeptide and carbohydrate chains.<sup>11</sup> Moreover, the antigenic specificity of these molecules is closely associated with the carbohydrate fraction.<sup>11-13</sup> Initial experiments revealed that the human colonic tumor-specific antigens demonstrated in our laboratory were mucoprotein in nature.<sup>8</sup> More recent enzyme digestion studies performed in our laboratory indicate that the antigenic determinants of the tumor-specific components are related to the polysaccharide constituents of the antigen molecules. In the case of the ragweed system, it has been established that aqueous ragweed extract is composed of a heterogenous mixture of proteins and polysaccharides<sup>14-16</sup> and that both types of constituents may serve as antigenic determinants.

The experimental findings suggest that it is possible to correlate the nature of the antigenic determinants of the various antigen-antibody systems with the effect of the sequence of erythrocyte sensitization on the hemagglutination titers obtained. Thus, it should be emphasized that the original BDB-hemagglutination technique described by Pressman and associates<sup>1</sup> was developed for use with

protein antigens which are coupled by this method to erythrocytes through stable covalent bonds. This firm binding occurs primarily through histidine and tyrosine residues. It is also known that substances containing carbohydrate or carbohydrate and phenolic constituents, such as bacterial polysaccharides and tannic acid, respectively, may coat red cells directly and nonspecifically.<sup>17, 18</sup> It may therefore be postulated that, in the case of protein- or peptide-containing antigens having carbohydrate or phenolic determinants, the addition of the antigen to the erythrocyte suspension prior to addition of the BDB could allow the determinant groups to be nonspecifically adsorbed onto the red cell surfaces.

At least two factors could be responsible for the marked reduction in the intensity of the hemagglutination reaction following the nonspecific adsorption of antigen. First, there would be a reduction in the number of antigenic molecules in solution available for binding through an azo linkage upon the addition of BDB. Perhaps of greater importance, however, would be the block interposed by the nonspecifically adsorbed antigen molecules between the red cell surface and the BDB. This block could function by either occluding the red cell binding sites usually involved in the azo linkage with BDB or alternatively could interfere with the approach of the BDB molecules to the erythrocyte surface through steric hindrance.

However, if the BDB and the erythrocytes were mixed prior to the addition of the antigen, the BDB molecules already coupled to the red cell surface would tend to link to the antigen molecules through the histidine and tyrosine groups of their protein or peptide portions. The free polysaccharide determinants would then be available to take part in a hemagglutination reaction upon the addition of homologous antiserum. This hypothesis would explain the results obtained with the chlorogenic acid-HSA conjugates, the A,B blood group-specific substances, and the tumor-specific antigens, all of which contain carbohydrate or carbohydrate and phenolic constituents in their antigenic determinant groups.

Since proteins do not appear to be nonspecifically adsorbed onto erythrocytes, protein antigenic determinants would not be blocked by adsorption onto red cell surfaces in the absence of BDB. In the presence of BDB, however, a protein antigenic molecule would be specifically coupled through a diazo linkage to the erythrocyte. The factors which determine whether the antigen molecule couples through its protein determinant group or through another portion of the protein structure include the relative concentrations of histidine and tyrosine in the various parts of the molecule, molecular spatial configuration, and chance contact. In any event, the coupling reaction would remain independent of the sequence of addition of the BDB and the antigenic material to the red cell suspension. This explanation would account for the fact that the hemagglutination titers in the BSA and tuberculin systems did not change when the BDB was added to the erythrocytes before rather than after the addition of antigen.

Since ragweed extract probably contains both protein and polysaccharide antigenic constituents, the positive hemagglutination reactions obtained in the ragweed system when BDB was added after the antigenic material were probably due to the interaction of antiprotein determinant antibodies with protein

determinant groups. The rise in titer observed when the sequence of addition was reversed may be best explained by the additive effect of antigen-antibody interactions involving both protein and polysaccharide antigenic determinants.

Thus, the application of BDB hemagglutination to systems of protein- or peptide-containing antigens which have phenolic and/or carbohydrate determinant groups requires that the erythrocytes be sensitized by the addition of BDB before the addition of the antigen. This finding may serve to explain the unusually low hemagglutination titers which have been recorded in certain complex protein-polysaccharide antigen-antibody systems where erythrocyte sensitization was performed by adding the BDB after the addition of the antigen.<sup>19</sup> It may also account for the failure, in certain cases, to demonstrate BDB-hemagglutination properties in antigen-antibody systems where precipitin reactions have been observed.<sup>20</sup>

The dual method of BDB-hemagglutination described in the present investigation may prove useful in studies designed to elucidate the chemical nature of the antigenic determinants of complex protein-polysaccharide substances. If the hemagglutination titers obtained with the two methods of erythrocyte sensitization are not significantly different, then the determinant groups are most likely protein in nature. If, however, a significant increase in titer occurs when the red cells are sensitized by adding the BDB before rather than after the addition of the antigen, then it is probable that the antigenic determinants are composed, in part at least, of the nonprotein constituents.

#### REFERENCES

1. Pressman, D., Campbell, D. H., and Pauling, L.: The Agglutination of Intact Azerythrocytes by Antisera Homologous to the Attached Groups, *J. Immunol.* **44**: 101, 1942.
2. Stavitsky, A. B., and Arquilla, E. R.: Micro Methods for the Study of Proteins and Antibodies. III. Procedure and Application of Hemagglutination Inhibition Reactions With Bis-diazotized Benzidine and Protein Conjugated Red Blood Cells, *J. Immunol.* **74**: 306, 1955.
3. Gordon, J., Rose, B., and Schon, A. H.: Detection of "Non-precipitating" Antibodies in Sera of Individuals Allergic to Ragweed Pollen by an *in Vitro* Method, *J. Exper. Med.* **108**: 37, 1958.
4. Cole, L. R., and Farrell, V. R.: A Method for Coupling Protein Antigens to Erythrocytes. I. Description of Method, *J. Exper. Med.* **102**: 631, 1955.
5. Schon, A. H.: The Detection and Nature of Nonprecipitating Antibodies in Allergic Sera, *in* Shaffer, J. H., LoGrippe, G. A., and Chase, M. W., editors: *Mechanisms of Hypersensitivity*, Boston, 1959, Little, Brown & Co., p. 61.
6. Arbesman, C. E., Rose, N. R., Kantor, S. Z., and Beede, R. B.: Immunologic Studies of Ragweed-Sensitive Patients, *J. ALLERGY* **31**: 317, 1960.
7. Turcotte, R., Freedman, S. O., and Schon, A. H.: A New Hemagglutination Procedure for the Diagnosis of Tuberculosis, *Am. Rev. Resp. Dis.* **88**: 725, 1963.
8. Gold, P., and Freedman, S. O.: Demonstration of Tumor-Specific Antigens in Human Colonic Carcinomata by Immunological Tolerance and Absorption Techniques, *J. Exper. Med.* **121**: 439, 1965.
9. Bariana, D. S., Krupey, J., Scarpati, L. M., Freedman, S. O., and Schon, A. H.: Chlorogenic Acid: Further Evidence for its Antigenic and Allergenic Activity, *Nature* **207**: 1155, 1965.
10. Pennell, R. B.: Fractionation and Isolation of Purified Components by Precipitation Methods, *in* Putnam, F. W., editor: *The Plasma Proteins*, Vol. I, Isolation, Characterization and Function, New York, 1960, Academic Press, Inc., p. 49.
11. Prankerd, T. A. J.: *The Red Cell*, Springfield, Ill., 1961, Charles C Thomas, Publisher, p. 23.
12. Morgan, W. T., and Watkins, W. M.: Neutralization of the Anti-H Agglutinin in Eel Serum by Simple Sugars, *Nature* **169**: 825, 1952.
13. Kabat, E. A., and Mayer, M. M.: *Experimental Immunochemistry*, ed. 2, Springfield, Ill., 1961, Charles C Thomas, Publisher, p. 861.

14. Lea, D. J., and Sehon, A. H.: The Chemical Characteristics of Non-dialyzable Constituents of Ragweed Pollen, *Internat. Arch. Allergy* **20**: 203, 1963.
15. Meacock, S. C. R., Freedman, S. O., and Sehon, A. H.: Characterization of the Dialyzable Constituents of the Aqueous Extract of Ragweed Pollen, *J. ALLERGY* **35**: 43, 1964.
16. Richter, M., and Sehon, A. H.: Studies on the Allergens of Ragweed Pollen, *J. ALLERGY* **31**: 111, 1960.
17. Boyden, S. V.: The Immunological Response to Antigens of the Tubercle Bacillus; Some Experimental Aspects, in Kallos, P., editor: *Progress in Allergy*, Basel, 1958, S. Karger, p. 149.
18. Boyden, S. V.: Absorption of Proteins on Erythrocytes Treated With Tannic Acid and Subsequent Hemagglutination by Antiprotein Sera, *J. Exper. Med.* **93**: 107, 1951.
19. Lowenstein, L., Cooper, B. A., Brunton, L., and Gartha, S.: An Immunological Basis for Acquired Resistance to Oral Administration of Hog Intrinsic Factor and Vitamin B<sub>12</sub> in Pernicious Anemia, *J. Clin. Invest.* **40**: 1656, 1961.
20. De Carvalho, S.: Clinical Immunological Studies of Natural Resistances Against Neoplasia, read before the 55th Annual Meeting of the American Association for Cancer Research, Chicago, Ill., April 11, 1964.