

IMMUNOLOGIC STUDIES OF RAGWEED-SENSITIVE PATIENTS

I. Specificity and Sensitivity of Hemagglutination Reactions

Carl E. Arbesman, M.D., Noel R. Rose, Ph.D., S. Zwi Kantor, M.D., and Robert B. Beede, M.D.,** Buffalo, N. Y.*

THE development of techniques to demonstrate antibodies in vitro in sera of allergic patients has been the goal of many investigators for several decades. As early as 1925, Coca and Grove¹ were unable to demonstrate precipitins in the sera of allergic persons, although these sera did contain the skin-sensitizing antibody. Many other workers in this field have confirmed this discrepancy. It was believed by many that the lack of precipitin in the allergic sera was due either to the fact that we were dealing with a univalent or incomplete antibody in ragweed-sensitive patients or to the fact that our methods of determining precipitins were too insensitive to demonstrate small amounts of this type of antibody. Cannon and Marchall² and Cohen and Weller,³ by using collodion particles coated with ragweed pollen extract, were able to show antibodies in vitro in some ragweed-allergic sera. This method was quite cumbersome and not readily reproducible. In more recent years, however, red blood cells instead of collodion particles have been successfully employed, and antigen can be either coated nonspecifically or linked chemically to these red blood cells. The addition of the specific antiserum produces a clumping of the red blood cells. This procedure was called indirect hemagglutination. It has been described as being 1,000 times more sensitive than the precipitin test.⁴

One method of hemagglutination was that first described by Boyden,⁵ who coated antigens on red blood cells that had been previously treated with tannic acid. Several investigators⁶⁻⁹ have used modifications of this method to measure antibodies in patients sensitive to pollens. For the most part, antibodies demonstrated by this method were found (in relatively low titers) primarily in the sera of patients who had received specific hyposensitization therapy. Feinberg and associates⁹ noted positive hemagglutination reaction in 21 per cent of untreated patients and 67 per cent of patients treated with pollen extract.

From the Allergy Research Laboratory, Buffalo General Hospital, and the Departments of Medicine and of Bacteriology and Immunology, University of Buffalo School of Medicine.

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*Research Fellow in Allergy, now in Tel Aviv, Israel.

**Research Fellow in Allergy, now in Utica, New York.

They also stated that there was elution of the antigen that was coated on the tannic-acid-treated red blood cells, which may have accounted for their relatively low titers.

Another method of hemagglutination, first described by Pressman and colleagues,¹⁰ consisted of an actual chemical linkage between the red blood cell and the antigen by means of bis-diazotized benzidine. This technique was applied and first used for pollen antigens by Stavitsky and Arquilla.¹¹ It was more thoroughly studied by Gordon and associates,¹² who reported that the sera of all ragweed-sensitive patients, either treated or untreated, had antibodies demonstrable by this method and that this test was specific.

Recently, Beede, Rose, and Arbesman¹³ used successfully the tanned-cell technique, as developed by Witebsky and Rose,¹⁴ with animal sera and danders as antigens.

The purpose of our study was to determine the specificity and relationships of both the modified tanned-cell and bis-diazotized-benzidine (BDB) techniques. We used ragweed pollen as the antigen, sera from ragweed-sensitive patients, and antiragweed rabbit sera as the source of antibodies.

METHODS AND MATERIALS

Sources of Antibodies.—Samples of blood were collected under sterile conditions from 113 ragweed-sensitive patients from private practice and from the Allergy Clinic of the Buffalo General Hospital. The sera were kept frozen until ready for use. An additional group of sera was obtained from “non-ragweed-sensitive” allergic persons. Three groups of control sera were likewise gathered for our studies from (1) the serologic laboratory (hospitalized “sick” patients), (2) the blood bank (healthy persons), and (3) patients who had serum sickness and high hemagglutination titers against horse serum.

Antiragweed rabbit sera were prepared by several different means in order to obtain adequate precipitin and hemagglutinin titers. At first alum-precipitated ragweed extracts were injected subcutaneously in several sites; if, after a period of three to four weeks, trial bleedings failed to show adequate antibody response, other courses of immunization were carried out. These included intravenous injections of the alum-precipitated extracts and/or the saline ragweed extracts given three times weekly over a four-week period. One to two weeks following the last intravenous injection, a trial bleeding was again performed. If the titer was adequate, the animal was bled out. In many instances, however, three to four courses of intravenous immunization were necessary.

We also employed a method in which equal parts of a 15 per cent saline extract of ragweed pollen and Freund's complete adjuvant (0.1 ml. of each) were injected into the four footpads of the rabbit. Again, trial bleedings, titer determinations, and further immunization by both the intravenous and the intradermal routes were employed, when needed, to obtain the desired antibody response.

Despite all these intensive methods of immunization, several animals were unable to develop sufficient antibodies.

Sources of Antigens.—A 15 per cent extract of defatted short ragweed pollen in buffered saline* (without phenol) was prepared by first treating ragweed pollen with petroleum ether in a Soxhlet apparatus until the distillate was colorless. This “defatted” material was then extracted with a buffered saline solution in a shaking machine for forty-eight hours at room temperature. After Buchner and Seitz filtration, the extract was bottled in 30 ml. vials and placed in the freezer. It was used in all the experiments to be reported in this and subsequent presentations.

A 15 per cent June grass extract was prepared in a similar manner.

Horse serum was obtained from a local slaughterhouse and kept frozen until ready for use.

Materials for Tanned-Cell Hemagglutination Test.—Human group O, Rh negative, red blood cells were taken in standard ACD† solution.

Tannic acid (C. P. Baker—not fluffy) stock solution, 0.5 Gm. in 100 ml. of distilled water (1:200) was prepared fresh each week. Dilutions for immediate use were made with buffered saline, pH 7.2.

The pH 7.2 buffered saline was prepared by adding 20.4 Gm. NaCl, 4.45 Gm. Na_2HPO_4 (anhydrous), and 1.3 Gm. KH_2PO_4 to 3,000 ml. of distilled water. This solution was checked by a pH meter and adjusted if necessary.

The pH 6.4 buffer was prepared by mixing 26.7 ml. of M/15 Na_2HPO_4 to 73.3 ml. M/15 KH_2PO_4 .

*Materials for Bis-Diazotized-Benzidine Method.*¹²—Normal rabbit red blood cells were collected from the ear vein into an equal volume of Alsever’s solution‡ and stored at 4° C. for as long as ten days. The red blood cells, when needed, were separated by centrifugation and washed three times with normal saline.

Normal rabbit serum was collected from the same rabbit that supplied the erythrocytes.

Bis-diazotized benzidine (BDB) was prepared by dissolving 0.23 Gm. of benzidine in 100 ml. of water, to which was added 0.75 ml. of 12 N hydrochloric acid. Next, 0.175 Gm. of NaNO_2 was dissolved in about 5 ml. of water, and this was slowly added to the solution. The reaction was allowed to proceed for thirty minutes with intermittent stirring. Aliquots of the solution were then placed into 2 ml. vials, quick frozen at -78° C. in a dry ice-acetone bath, and stored at -20° C.

The 0.15 M pH 7.3 phosphate buffer was prepared by mixing 2.5 ml. of 0.15 M Na_2HPO_4 with 49 ml. of 0.15 M KH_2PO_4 .

Method of Tanned-Cell Hemagglutination Test.—

1. *Washing red blood cells:* To 2.0 ml. of human group O, Rh negative, red blood cells was added 20 ml. of buffered saline, pH 7.2. The tube was

*Buffered saline solution:

Sodium chloride	0.5%
Sodium bicarbonate	0.825%.

†ACD = Acid citrate dextrose (Baxter).

‡Alsever’s solution:

Glucose	2.05
Sodium citrate	0.8
Sodium chloride	0.42
Distilled water to	100 ml.

inverted gently two to three times and then centrifuged for five minutes at 1,500 r.p.m. in an International refrigerated centrifuge, Model PR-2 with Head #284. The supernatant was removed, and the same process was repeated. The final washing was performed in a graduated tube with only 10 ml. of pH 7.2 buffer. The supernatant was again removed, and a 2.5 per cent suspension of red blood cells with buffered saline, pH 7.2, was prepared.

2. *Tanning red blood cells:* To one volume of 2.5 per cent red blood cell suspension was added an equal volume of tannic acid 1:20,000, diluted fresh for each experiment with pH 7.2 buffered saline from the 1:200 stock solution. This mixture was incubated for ten minutes in a 37 degree water bath and then centrifuged for five minutes at 1,000 r.p.m. The supernatant was removed and the packed red blood cells were resuspended with two volumes of buffered saline, pH 7.2, and centrifuged in the same manner as before. This process of washing the tanned cells was repeated once more, but with normal saline instead of buffered saline, and the supernatant was removed.

3. *Coating red blood cells with antigen:* The packed, washed, tanned red blood cells were resuspended by the addition of a mixture of equal volumes of pH 6.4 buffered saline and short ragweed extract (15 per cent defatted saline) diluted in 1 to 5 with buffered saline, pH 7.2. This red blood cell-antigen mixture was incubated for fifteen minutes in a 37° C. water bath and then centrifuged, as described previously, for five minutes. The supernatant was removed, and the red blood cells were resuspended with two volumes of 0.5 per cent normal rabbit serum (diluted with buffer saline pH 7.2) and centrifuged as before. This same process was repeated, and the cells were finally resuspended with 0.5 per cent normal rabbit serum to 1.25 per cent (approximately 4.5 ml.).

4. *Dilution of antisera:* Serial twofold dilutions (volume, 0.5 ml.) of antisera were made with 1 per cent normal rabbit serum. A separate pipette was used for each dilution.

5. *Test:* Tanned, coated red blood cells (1.25%) (volume, 0.1 ml.) were added to each tube of the serial dilutions of antisera (volume, 0.5 ml.) and to a control tube with normal serum. Another control consisted of tanned uncoated cells added to antisera.

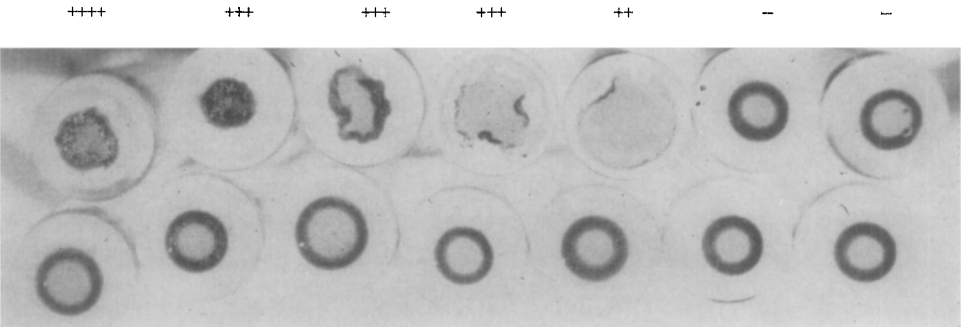
The mixtures are shaken vigorously, the cells were allowed to sediment at room temperature for two hours, and the patterns were read. The tubes were again shaken and kept in the icebox overnight, and the patterns were read again.

6. *Reading of patterns:* The degree of hemagglutination was recorded from 4 plus to 0. A 4 plus reaction consisted of marked folding noted at the bottom of each tube. A reaction with less folding was considered a 3 plus reaction, and one with less tightly agglutinated cells was recorded as 2 plus. A 1 plus reaction presented a diffuse granular appearance, and a negative reaction had the appearance of a doughnut or ring (Fig. 1).

The difference in techniques between our method and those described by Feinberg and Flick and by Stavitsky are illustrated in Table I. We have

observed that the slight variations in technique from the Feinberg-Flick method gave us regularly reproducible results and no evidence of elution of antigen.

Bis-Diazotized-Benzidine (BDB) Method.—The same technique as that described by Gordon and co-workers¹² was employed in our studies, except that we used a different antigen. In preliminary studies it was determined that a 1:40 dilution (with pH 7.2 buffered saline) of a 15 per cent saline extract of defatted short ragweed pollen gave hemagglutination titers equivalent to their water-soluble ragweed extract 0.75 per cent. One other slight difference is that our test tubes were 12 mm. in diameter, whereas Gordon and associates used 10 mm. tubes.



Neg. Neg. Neg. Neg. Neg. Neg. Neg.
Fig. 1.—Tanned-cell hemagglutination patterns. (For method of reading patterns, see text.)

TABLE I. TANNED-CELL HEMAGGLUTINATION METHODS FOR RAGWEED ANTIGENS			
	FEINBERG AND FLICK	STAVITSKY AND ARQUILLA	PRESENT METHOD
1. Red blood cells	Human group O, Rh negative, in Alsever's solution	Sheep	Human group O, Rh negative, in ACD
2. Washing red blood cells	Normal saline; then resuspended in 7.2 buffered saline	Normal saline	7.2 buffered saline
3. Coating red blood cells	Red blood cells first resuspended in pH 6.4 buffer, then antigen added	Red blood cells resuspended in buffered saline; then added to mixture of 1 volume of antigen and 4 volumes of 6.4 buffer	Red blood cells suspended in mixture of equal volumes of antigen and pH 6.4 buffer
4. Incubation	15 minutes at 37° C.	10 minutes at room temperature	15 minutes at 37° C.
Washing coated red blood cells	Normal rabbit serum 1:200 in normal saline twice	Normal rabbit serum 1:100 in normal saline once	Normal rabbit serum 1:200 in 7.2 buffered saline twice
Resuspension	Normal rabbit serum 1:200 in normal saline 1.8%	Normal rabbit serum 1:100 in normal saline 2.5%	Normal rabbit serum 1:200 in 7.2 buffered saline 1.25%
5. Volume of anti-sera used	0.1 c.c.	0.5 c.c.	0.5 c.c.
6. Volume of coated cells added	0.05 c.c.	0.05 c.c.	0.1 c.c.

The recording of the patterns with the BDB technique was different from the tanned-cell patterns. A negative test was recorded only when the cells formed a compact button on the bottom of the tube. A 1 plus reaction was evidenced by a small doughnut or ring. A 2 plus reaction had a very large ring. A 3 plus and a 4 plus reaction consisted of folding of the edges and strong agglutination of cells. These varying degrees of reaction are shown in Fig. 2.

Tanned-Cell Method for Horse Serum Antigen.—A modified technique, described in detail in another publication,¹⁵ was employed with horse serum as the antigen to coat the red blood cells.

Inhibition Tests.—

1. Varying dilutions of short ragweed pollen extract in 1 per cent normal rabbit serum (0.5 ml.) were added to 0.1 ml. of antiragweed rabbit serum. This was set up in several rows, with a different dilution of the antiragweed rabbit serum in each row. The mixtures were shaken and remained at room temperature for two hours. Tanned, coated red blood cells (0.1 ml.) were added to each tube. The tubes were shaken, and the patterns were recorded as before.

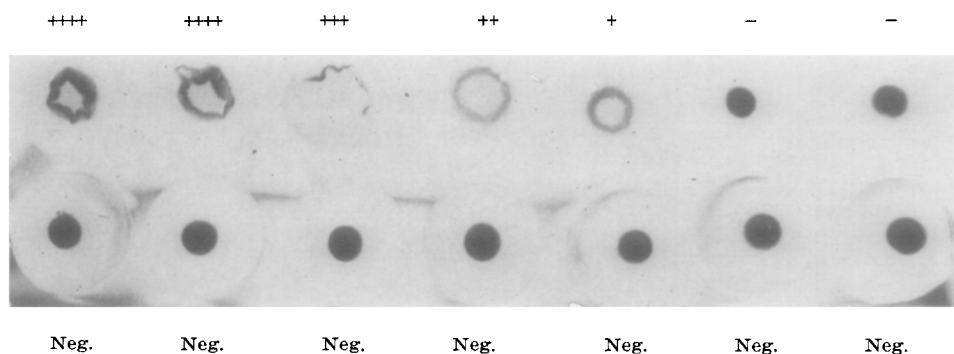


Fig. 2.—BDB hemagglutination pattern.

A similar technique of mixing varying dilutions of ragweed extract and antiragweed rabbit serum was used for the BDB method to demonstrate specific inhibition.

Various antigens, other than ragweed, were likewise used in these inhibition experiments.

2. Another type of inhibition test consisted of mixing equal parts of the pollen antigen and antirabbit serum. The mixture was incubated at room temperature for one hour, and the serial dilutions with 1 per cent normal rabbit serum were made of this mixture. Ragweed-coated tanned red blood cells were then added to these various tubes, and the results were recorded as previously described. The specificity of the test was determined by using June grass pollen extract as an inhibiting antigen and also for coating red blood cells.

3. Absorption tests were also carried out. Human serum from ragweed-sensitive patients (1.5 ml.), diluted 1:5 with 1 per cent normal rabbit serum, was added to 0.25 ml. of packed red blood cells treated with bis-diazotized benzidine

and linked to ragweed extract 1:40. This mixture was shaken and incubated at room temperature for one hour. Following centrifugation (as with the regular BDB technique), the supernatant was removed. An aliquot was saved, and the remainder of the supernatant was again exposed to red blood cells linked to ragweed extract by BDB. The same process as with the first absorption was repeated a total of five times. The aliquot following each absorption was then set up in duplicate serial dilutions with 1 per cent normal rabbit serum. BDB and tanned-cell hemagglutination tests were then performed by the technique described earlier for the unabsorbed serum and the various aliquot samples.

This same procedure was performed, absorbing the human ragweed serum with tanned red blood cells coated with ragweed extract.

Controls for this type of test consisted of absorbing the human serum with red blood cells treated with BDB (or tannic acid) and linked to horse serum rather than to ragweed antigen and absorbing with red blood cells untreated and uncoated.

RESULTS

Hemagglutination Tests.—

Antiragweed rabbit sera: The sera of twenty-four rabbits, immunized by various means as previously described, were tested for their hemagglutination activity. Table II shows the titers obtained in several of these animals by hemagglutination and by the usual precipitin tests. Seventeen of the twenty-four animals developed hemagglutination titers, ranging from a low of 2,000 to a high of 256,000. Although there was a close correlation (within two dilution steps) in titers obtained by the tanned-cell and BDB methods in most instances, several sera had high BDB and low tanned-cell titers and some had high tanned-cell and low BDB titers.

TABLE II. HEMAGGLUTINATION AND PRECIPITIN TITERS OF ANTIRAGWEED RABBIT SERA

RABBIT NO.	TANNED-CELL TITERS*	BDB TITERS*	PRECIPITIN END POINTS†
4	128,000	256,000	1-1,000
12	32,000	256,000	1-100
24	128,000	128,000	1-10,000
44	16,000	32,000	1-10,000
388	4,000	16,000	1-1,000
500	32,000	32,000	1-10
386	4,000	2,000	1-10
407	16,000	2,000	1-1,000
64	32,000	128,000	1-1,000
413	8,000	16,000	1-100

*Reciprocal of highest dilution of antiserum which showed positive hemagglutination reaction.

†Highest dilutions of antigen with constant amount of undiluted antiserum, which showed a positive precipitin reaction.

Human sera: The sera of 113 hay fever (ragweed-sensitive) patients were tested by both techniques of hemagglutination, as were sixty-nine sera from the serologic laboratory (hospitalized patients), ninety-five sera from the blood

bank (healthy persons), sera from forty-nine patients who had serum sickness and high titers by hemagglutination with horse serum, and sera from nineteen allergic patients supposedly nonallergic to ragweed pollen. Table III shows the results obtained with these various groups of sera. Of the 113 ragweed-sensitive patients, twenty-one had never received any type of previous hypo-sensitization therapy; of the remainder, none had received any previous treatment for at least two to four months. Almost 100 per cent of these sera showed positive BDB hemagglutination titers, and 87 per cent showed positive titers by the tanned-cell method. There was no apparent difference between the sera of the untreated and the previously treated patients. If, however, we considered a significant titer for the BDB test to be 1-80 or greater and for the tanned-cell test, 1-20 or greater, then the percentage of positives would be considerably lower. (Table III—figures in parentheses.)

TABLE III. HEMAGGLUTINATION TITERS

SOURCE OF SERA	BIS-DIAZOTIZED-BENZIDINE METHOD			TANNED-CELL METHOD		
	NUMBER OF PATIENTS	NUMBER POSITIVE	PER CENT POSITIVE	NUMBER OF PATIENTS	NUMBER POSITIVE	PER CENT POSITIVE
Blood bank	92	42 (18)*	46 (19)	95	13 (10)†	14 (11)
Serology laboratory	69	35 (12)	50 (17)	63	12 (12)	19 (19)
Serum sickness patients	38	30 (14)	80 (37)	49	11 (8)	22 (16)
"Allergic" controls	16	12 (5)	75 (31)	19	6 (4)	32 (21)
<i>Ragweed-sensitive patients</i>						
A‡	92	90 (71)	98 (77)	92	80 (63)	87 (68)
B§	21	21 (14)	100 (67)	21	18 (11)	85 (57)

*BDB titers of 80 or greater.

†Tanned-cell titers of 20 or greater.

‡Ragweed-sensitive patients who had received specific hyposensitization therapy in previous years.

§Ragweed-sensitive patients who had never received specific hyposensitization therapy.

In the allergic group of patients who supposedly were not allergic to ragweed, there was still a high percentage of positive titers (75 per cent and 32 per cent by the BDB and tanned-cell methods, respectively). However, all of these patients were retested with a stronger ragweed extract (10,000 PTA/n) intradermally, and it was found that three patients who demonstrated positive hemagglutination titers by the tanned-cell method also had positive skin tests, and two patients with positive BDB titers likewise had positive skin tests. Hence, if we were to eliminate these patients from the group, the positive hemagglutination titers would be 19 per cent and 71 per cent by the tanned-cell and BDB methods, respectively.

The incidence of positive reactions in the control groups (the group of sera obtained from hospitalized patients, from the serologic laboratory, from blood donors from the blood bank, and from patients who had serum sickness

and a high hemagglutination titer against horse serum) are likewise shown in Table III. It will be noted that there is a higher incidence of positive reactions in both groups by the BDB method than by the tanned-cell method. Hence, in summary, in all the various control groups of sera tested, the BDB test showed positive reactions in approximately 50 per cent, whereas the tanned-cell test revealed positive reactions in only about 18 per cent.

Comparative titers of pretreatment sera of ragweed-sensitive patients: It can be seen that in some instances the BDB titer may be relatively high and the tanned-cell titer low for the same serum (Fig. 3). Of these seventy-two male and forty-one female subjects tested, the BDB titer was higher in seventy-five (67 per cent), the tanned-cell titer was higher in eighteen (16 per cent), and the same titers were obtained in another eighteen (16 per cent).

COMPARISON OF 113 PRE-TREATMENT SERA

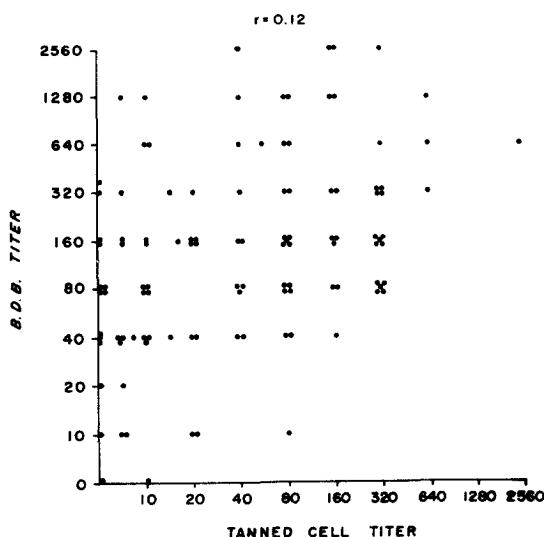


Fig. 3.—The actual hemagglutination titers as determined by the bis-diazotized-benzidine method and the tanned-cell technique in the same samples of sera from ragweed-sensitive patients prior to therapy. The numbers are the reciprocal of the highest dilution of the patient's sera that produce a positive hemagglutination reaction.

r = Correlation coefficient, which in this instance means that the value of 0.12 is not statistically significant.

Relationship of age distribution and hemagglutination titers: The number of patients in each age group and the average hemagglutination titers as determined by the BDB and tanned-cell tests are shown in Table IV. It appears that

TABLE IV. SUMMARY OF TITERS* ACCORDING TO AGE DISTRIBUTION

AGE	NUMBER OF PATIENTS	GEOMETRIC MEAN TITER	
		TANNED-CELL	BDB
0-10	6	56.6	320
11-20	23	39.4	165
21-30	31	37.1	125
31-40	24	23.1	109
41-50	14	32.7	160
51-60	10	18.2	211

*Reciprocal of highest dilution of serum which produced positive hemagglutination.

there is a gradual reduction in BDB titers with age up to the age of 40, and then there is an increase. When these findings were studied statistically, however, there was no real difference in the mean titers and the age of the patient.

Relationship of duration of symptoms and hemagglutination titers: The sera studied were obtained from patients who had had symptoms for one to fifty years. The duration of symptoms in years and the number of patients in each group, along with their respective titers, are listed in Table V. No apparent relationship was noted between the duration of symptoms and geometric mean titers measured by either the tanned-cell or the BDB method.

TABLE V. COMPARISON OF PRETREATMENT TITERS* TO DURATION OF SYMPTOMS (NUMBER OF YEARS OF PREVIOUS SYMPTOMS)

PREVIOUS SYMPTOMS (NUMBER OF YEARS)	NUMBER OF PATIENTS	GEOMETRIC MEAN TITER	
		TANNED-CELL	BDB
0-5	27	35.5	132.6
6-10	27	28.3	152.0
11-15	21	20.9	160.0
16-20	8	46.3	67.0
21-25	16	66.8	153.0
Over 25	12	19.2	151.0

*Reciprocal of highest dilution of serum which produced positive hemagglutination.

Relationship of number of years of previous specific hyposensitization therapy to the hemagglutination titers: Twenty-one of the 113 patients had never had previous therapy, whereas twenty-three had undergone treatment for six to ten years and eight patients had received treatment for more than ten years. As can be seen from Table VI, there is no apparent correlation between the number of years of previous treatment and either the BDB or the tanned-cell titers. It appears that the sera of those patients with no previous treatment had lower titers, but here again the finding is not statistically valid.

TABLE VI. COMPARISON OF NUMBER OF YEARS OF PREVIOUS TREATMENT TO TITERS*

NUMBER OF YEARS OF PREVIOUS TREATMENT	NUMBER OF PATIENTS	GEOMETRIC MEAN TITER	
		TANNED-CELL	BDB
0	21	12.8	96.6
1	18	34.7	205.9
2	11	73.6	173.6
3	19	66.3	158.2
4-5	11	42.7	150.1
6-10	23	27.3	124.3
Over 10 years	8	12.3	190.3

*Reciprocal of highest dilution of serum which produced positive hemagglutination.

Relationship of time interval between last injection of pollen extract and the hemagglutination titers: Twenty-one patients had received no previous injections, and fourteen had received their last injections more than two years prior to the pretreatment bleeding. Eight patients, however, had been given injections two to four months prior to the time their sera were obtained. As can be seen from Table VII, the titers of sera obtained from patients who had never received specific hyposensitization therapy are lower than those of sera from patients who had previously received treatment. There were significant

TABLE VII. RELATION OF TIME OF LAST INJECTION OF ANTIGEN TO PRETREATMENT TITER*

MONTHS SINCE LAST INJECTION	NUMBER OF PATIENTS	GEOMETRIC MEAN TITER	
		TANNED-CELL	BDB
Not previously injected	21	13.8	94.4
More than 24	14	22.5	113.1
9 to 24	13	26.0	110.1
8	12	36.9	124.7
7	10	44.1	139.3
6	18	33.6	345.6
5	15	70.2	192.5
2 to 4	8	46.3	103.7

*Reciprocal of highest dilution of serum which produced positive hemagglutination.

differences between the titers of sera from those patients who received their last injection five and six months (tanned-cell and BDB, respectively) previous to taking of the samples and those from patients who had received no treatment prior to the time their sera were secured.

Specificity of Hemagglutination Tests.—

1. Antiragweed rabbit sera were tested by these two techniques. The cells were coated with another pollen antigen, June grass. It is interesting to note (Table VIII) that there was some evidence of hemagglutination when June

TABLE VIII. TANNED-CELL HEMAGGLUTINATION TEST (CROSS REACTIONS BETWEEN RAGWEED AND JUNE GRASS)

	RABBIT ANTISERA			
	ANTIRAGWEED	ANTI-JUNE GRASS	ANTITIMOTHY	ANTIPLANTAIN
<i>Red Blood Cells Coated With Ragweed Extract</i>				
Titer	5,000	80	Not done	Not done
<i>Red Blood Cells Coated With June Grass Extract</i>				
Titer	640	10,000	5,000	640

See text for method.

TABLE IX. INHIBITION OF HEMAGGLUTINATION REACTION OF ANTIRAGWEED RABBIT SERUM (No. 4) AND RED BLOOD CELLS COATED WITH RAGWEED AND JUNE GRASS EXTRACTS BY THE CORRESPONDING AND HETEROLOGOUS ANTIGENS

SUBSEQUENT DILUTION OF SERUM-ANTIGEN MIXTURES IN 1 PER CENT NORMAL RABBIT SERUM	ANTIRAGWEED RABBIT NO. 4 SERUM DILUTION 1:50					
	RED BLOOD CELLS COATED WITH RAGWEED			RED BLOOD CELLS COATED WITH JUNE GRASS		
	JUNE GRASS EXTRACT	RAGWEED EXTRACT	NORMAL RABBIT SERUM (CONTROL)	JUNE GRASS EXTRACT	RAGWEED EXTRACT	NORMAL RABBIT SERUM (CONTROL)
1:100	++++	--	+++	--	--	+++
1:200	++++	--	+++	--	--	+++
1:400	++++	--	+	--	--	+
1:800	++	--	+	--	--	+
1:1,600	+	--	+	--	--	+
1:3,200	+	--	+	--	--	+
1:6,400	+	--	+	--	--	+
1:12,800	+	--	+	--	--	--
1:25,600	+	--	+	--	--	--
1:51,200	--	--	--	--	--	--
1:102,400	--	--	--	--	--	--
1:100 (control)	--	--	--	--	--	--

See text for method.

grass was used as the antigen. Inhibition tests using anti-June grass and anti-ragweed rabbit sera and the corresponding homologous and heterologous antigens were also performed (Table IX). It can be observed that the ragweed antigen completely inhibits the reaction between the antiragweed serum and the red blood cells coated with both June grass and ragweed antigen, whereas the June grass antigen inhibits only the reaction with the June grass-coated cells but not with the ragweed-coated cells.

2. Red blood cells were coated with June grass and tested with the human ragweed sera. Only one patient, who had positive skin tests and was clinically sensitive to grasses, showed positive hemagglutination titers (Table X).

TABLE X. CROSS REACTIONS BETWEEN RAGWEED AND JUNE GRASS IN HEMAGGLUTINATION (TANNED-CELL) REACTIONS IN RAGWEED-SENSITIVE SERA

PATIENT	TANNED-CELL TITER	
	RAGWEED	JUNE GRASS
B. G.	160	0
M. B.	320	0
M. F.	40	40*
B. S.	40	0
J. D.	5	0
W. K.	160	0
G. B.	80	0

*Also had grass symptoms and positive skin test.

TABLE XI. SPECIFIC INHIBITION BY HOMOLOGOUS ANTIGEN OF HEMAGGLUTINATION TEST (TANNED-CELL)

DILUTIONS OF INHIBITING RAGWEED EXTRACT (0.5 ML.)	DILUTIONS OF ANTIRAGWEED RABBIT SERUM (0.1 ML.)		
	1:100	1:200	1:400
1:1,600	--	--	--
1:3,200	+	--	--
1:6,400	+	--	--
1:12,800	+	+	--
1:25,600	+	+	+
0	+	+	+
(1:10 normal rabbit serum)	--	--	--

TABLE XII. SPECIFIC INHIBITION BY HOMOLOGOUS ANTIGEN (RAGWEED POLLEN EXTRACT), BDB METHOD

DILUTIONS OF INHIBITING ANTIGEN RAGWEED POLLEN EXTRACT IN NORMAL RABBIT SERUM 1:100	DILUTIONS OF ANTIRAGWEED RABBIT SERUM					
	1:10	1:20	1:40	1:80	1:160	1:320
1:100	--	--	--	--	--	--
1:200	--	--	--	--	--	--
1:400	+	--	--	--	--	--
1:800	+	--	--	--	--	--
1:1,600	+	+	--	--	--	--
1:3,200	+	+	+	--	--	--
1:6,400	+	+	+	+	--	--
1:12,800	+	+	+	+	+	--
1:25,600	+	+	+	+	+	--
1:51,200	+	+	+	+	+	+
1:204,800	+	+	+	+	+	+
0	+	+	+	+	+	+
1:10	--	--	--	--	--	--

Inhibition Tests.—Specific inhibition tests, as described in our discussion of methods and materials, were employed with both the BDB and the tanned-cell tests. The results of inhibition tests are shown in Tables XI and XII. It can be seen that there is a specific inhibition with the homologous antigen and that it falls in a linear pattern. As little as 1:1,600 dilution of the 15 per cent ragweed pollen antigen is required to prevent the hemagglutination of a 1:100 dilution of antiragweed rabbit serum.

Absorption Tests.—Inasmuch as there was very little correlation between the titers of the human sera determined by the BDB and tanned-cell methods, we considered the possibility that two different types of antibody were being measured, due perhaps to the fact that different fractions of antigen (pollen extract) attach to a tanned cell or linked by bis-diazotized benzidine to the red blood cells. Sera from four different patients were studied. Two of these sera had high BDB titers and relatively low tanned-cell titers. The other two sera had higher tanned-cell titers and lower BDB titers. An example of an absorption test is shown in Table XIII.

As can be seen, the coated tanned cells absorb out all the antibodies demonstrable in tanned cell tests and benzidine in one or at most two absorptions, but the BDB antibody requires three or sometimes four absorptions before complete neutralization is noted. The converse experiment was performed; that is, the same human antiserum was absorbed by red blood cells linked to ragweed extract by bis-diazotized benzidine. Aliquots of each absorption were then tested by both the BDB and the tanned-cell methods. In these experiments the BDB-coated cells were more effective in removing the antibody and neutralized both the tanned cells and the bis-diazotized benzidine in one or two absorptions. The results suggest that the antibodies playing the primary role in the two types of tests may not be the same.

DISCUSSION

Almost all the sera from ragweed-sensitive patients showed positive hemagglutination titers by either the BDB or the tanned-cell method. This would indicate that some type of antibody against ragweed is measured by these techniques. The inhibition tests have shown the reaction to be specific. Other pollens cannot produce the same effect in persons sensitive only to ragweed.

Unfortunately, we found no correlation between the BDB and tanned-cell titers in the same sera. This difference in results could be due to the fact that other antigen-antibody reactions play the decisive roles in the two types of tests. The absorption tests did not clearly establish this point, since absorption of the patient's sera with either BDB- or tanned-cell-treated cells eventually removed all the antibody detectable by either method. However, all the antigens may have been coated on the red blood cells, but it may not necessarily have taken a complete part in the antigen-antibody reaction as evidenced by visible hemagglutination.

Our studies revealed a much higher incidence of positive results in various control groups tested by bis-diazotized benzidine than reported by Gordon and

associates,¹² who obtained negative reactions in all of the twenty-two "normal" sera they tested. We followed exactly the methods described by these workers. Through the kind cooperation of Dr. Bram Rose, two of us went to the Montreal Laboratory for several days to observe, first hand, the details of this test. We were able to reproduce their results. We exchanged blind samples of sera in each of the two laboratories, and the results obtained were quite comparable. The one difference in our methods is the antigen involved. We found that the 1:40 dilution of our 15 per cent defatted saline extract gave results equivalent to the water soluble extract (WSR) which Gordon and colleagues used.

We are convinced, therefore, that our technique is correct and that our findings are valid and would indicate that in our hands BDB method has a lower specificity than does the tanned-cell technique. Patients with a positive tanned-cell titer are more likely to be ragweed sensitive than those with a positive BDB titer. Only 14 per cent of our random control group showed positive tanned-cell titers.* It was noted that there was no significant difference in the percentage of positive reactions between the sera obtained from the blood bank (supposedly healthy persons) and the sera obtained from the serologic laboratory (hospitalized sick patients). This would indicate that disease processes had no effect on the hemagglutination titer produced by ragweed-coated or -linked cells. However, there was increased evidence of positive reactions, especially apparent by the BDB method, in those patients with such hypersensitive states as serum sickness or atopic disease.

Moreover, we found that although higher titers were usually obtained by the BDB method, the tanned-cell method seemed to be more reproducible and less likely to give the inconsistent results that we sometimes found by the BDB method. There was no evidence of elution of antigen from the tanned red blood cells, as was reported by Feinberg and Flick. This could be explained by either the different antigen used for coating the cells or the slight but significant changes in our methods of performing this test.

There is no correlation between the age of the ragweed-sensitive patient and the hemagglutination titer of his serum, the length of time the patient had symptoms, or the amount of previous treatment. However, the titers were definitely higher in those patients who had received injections five to six months previously, as compared to those patients who had never had specific treatment. This type of antibody which we measured by these methods is present in almost all ragweed-sensitive patients, regardless of age, duration of symptoms, or number of years of treatment. Furthermore, as will be reported in our next article, there was a definite increase in these antibody titers following intensive pollen therapy.

SUMMARY AND CONCLUSIONS

1. The sera from patients with ragweed sensitivity, both treated and untreated, contain substances which, when added to red blood cells coated or linked

*Although the patients from whom random serum samples were obtained were not interviewed or skin-tested for ragweed sensitivity, it is difficult to conceive that 46 per cent would be sensitive to ragweed as determined by the BDB technique. A more realistic figure of 14 per cent positive, as shown by the tanned-cell method, could conceivably have been from ragweed-sensitive patients.

to ragweed extract, cause a clumping of these cells (indirect hemagglutination).

2. Two methods, namely, the tanned-cell and bis-diazotized-benzidine methods, are suitable for demonstrating the particular antigen-antibody reaction. The results obtained by the two methods do not appear to correlate.

3. The BDB method usually gives higher titers than does the tanned-cell method in the sera of ragweed-sensitive patients. However, it also gives positive reactions to a much greater percentage of "random controls."*

4. The tanned-cell method, in our experience, is reliable, reproducible, and simpler to execute than is the BDB technique.

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*After submitting this article for publication, we received thirty-five sera from patients living in Israel who had never been exposed to ragweed pollen. Only one of these sera showed a positive hemagglutination titer by the tanned-cell technique in a dilution of 1:10. However, twenty-four of the thirty-five sera produced positive titers by the BDB method. Five of these thirty-five sera had titers greater than 1 to 40.