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1. Gordon, R. B., Kraft, S. C., Jones, R. J., J. Lab. and Clin. Med., 1953, v41, 583.
2. Rosenheim, O., Webster, R. A., J. Biol. Chem., 1941, v35, 920.
3. Jones, R. J., Kraft, S. C., Huffman, B. A., Balter, E. L., Gordon, R. B., Circ. Res., 1953, v1, 530.
4. Peterson, D. W., Proc. Soc. Exp. Biol. and Med., 1951, v78, 143.
5. Pollak, O. J., Circulation, 1953, v7, 702.
6. Best, M. M., Duncan, C. H., Van Loon, E. J., Wathen, J. D., ibid., 1954, v10, 201.
7. Katz, L. N., Stamler, J., Experimental Atherosclerosis, Charles C Thomas, Springfield, Ill., 1953.
8. Schoenheimer, R., Sperry, W. M., J. Biol. Chem., 1934, v106, 754.
9. Zilversmit, D. B., Davis, A. K., J. Lab. & Clin. Med., 1950, v35, 155.
10. Jones, R. J., ibid., 1956, v47, 261.
11. Jones, R. J., Reiss, O. K., Balter, E. L., Cohen, L., Proc. Soc. Exp. Biol. and Med., 1957, v96, 442.
12. Fisher, H., Weiss, H. S., Griminger, P., ibid., 1963, v113, 415.
13. Pollak, O. J., Circulation, 1953, v7, 696.
14. Pick, R., Stamler, J., Rodbard, S., Katz, L. N., ibid., 1952, v6, 276.
15. Hernandez, H. H., Peterson, D. W., Chaikoff, I. L., Dauben, W. G., Proc. Soc. Exp. Biol. and Med., 1957, v96, 498.
16. Davis, W. W., Trans. N. Y. Acad. Sci., 1955, v18, 123.

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A New Method for Detection of "Immune" Type of Anti-B Antibody.* (30319)

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Winstanley *et al*(1) and Konugres *et al*(2) established a method for detection of the "immune" anti-A antibody using certain pig red blood cells (A^p), and demonstrated the antibody by agglutination and hemolysis of the A^p red cells which possess human A-like antigen. Tovey *et al*(3) employed A^p red cells for prenatal diagnosis of the hemolytic disease of the newborn due to ABO incompatibility. These investigators used the method of antibody-absorption (A-A test); that is, the absorption of the immune anti-A antibody, but not the naturally occurring antibody with A^p red cells. Subsequently Yokoyama *et al*(4) and Polley *et al*(5) showed the usefulness of the A^p system for detection of immune anti-A antibody. Recently the A^p antigen was classified into 3 classes and the A^{p1} red cells were found to detect "immune" anti-A activity with a more definite reaction by agglutination and hemolysis than the A^{p2} and A^{p3} red cells(6). Yokoyama and Fudenberg(7) also reported that the anti-A^p activity occurred in the γ_2 globulin (IgG), γ_1A (IgA) and β_2M (IgM) fractions.

A similar method for detecting the immune anti-B antibody, which is different from naturally occurring anti-B antibody, has long been sought, with the intention of utilizing the method in pre- and post-natal diagnosis of hemolytic disease of the newborn due to an incompatible pregnancy involving the B antigen. The present paper describes a new method for detecting the immune anti-B antibody.

Materials and methods. Blood samples from 30 New Zealand white rabbits, between the ages of 2 and 7 months, were mixed with an equal volume of Alsever's solution. Prior to use the red cells were washed 4 times with physiological saline. The red cells from each rabbit were tested against blood grouping sera developed against rabbit rbc isoantigens.† A 2% red cell suspension in saline was mixed with an equal volume of antiserum and incu-

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† Anti-A, anti-D and anti-F obtained through the courtesy of Dr. Carl Cohen, Dept. of Biology, Western Reserve Univ., Cleveland, Ohio.

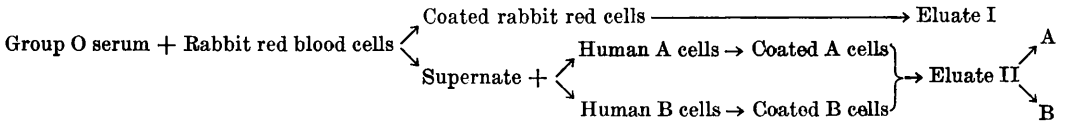

FIG. 1. Preparation of eluates for detection of cross-reacting antibody.

bated at 23°C for 30 minutes. Agglutination was determined after centrifugation.

Six each of human group O and group A pre-immunization sera from normal individuals, and group O and group A post-immunization sera from persons who had received injections of soluble specific blood group substance B, were used for demonstration of the immune anti-B antibody. Six each of human group O and group B pre-immunization sera from normal individuals, and group O and group B post-immunization sera from subjects immunized with soluble blood group substance A, were used as controls. All sera were obtained from Dr. William L. Epstein, Dept. of Dermatology, University of California, San Francisco Medical Center. Serum samples also were obtained from 2 group O mothers who had recently had group A infants with hemolytic disease, and from 2 group O mothers who had group B infants with hemolytic disease. The maternal sera were kindly supplied by the U. S. Tripler General Hospital, Honolulu.

All sera were inactivated at 56°C for 30 minutes and were absorbed with 2 volumes of washed, packed rabbit red cells at 23°C for 30 minutes. In general, heteroagglutinins were removed by a one-absorption process with fresh rabbit red cells. If necessary, the absorption experiments were repeated in the same manner until the serum did not react with the rabbit red cells which were used for absorption.

The absorbed sera were tested against human group A and group B red cells by direct saline agglutination at 23°C and also by the ficin method(8) at 37°C. In some experiments, the indirect antiglobulin test was also performed. The antibody titrations were carried out by 2-fold serial dilutions of serum with saline.

Antibody was eluted from rabbit red cells which had been absorbed with human sera by the heat elution method(9). The antibody also was eluted by the same method from human group A and B red cells which had been absorbed with human sera. These human sera were previously absorbed with rabbit red cells. Fig. 1 shows a diagram of the preparation of the eluates. The eluates were tested with human group A and group B red cells by the saline and ficin methods for demonstration of cross-reacting antibody.

Hemolysin tests were carried out using a 2% barbital-buffered saline suspension of human group A or group B red cells with absorbed sera. Equal volumes of ABO compatible fresh human group A or group B sera were used as complement, and were added to a mixture of serum and group A or group B red cell suspensions. The mixture was incubated at 37°C for one hour and examined for hemolysis after centrifugation.

Sera were fractionated by a method of Levy *et al*(10) using diethylaminoethyl (DEAE)-cellulose stepwise elution column chromatography. Serum samples were dialyzed against 0.01 M sodium phosphate buffer, pH 8.1, and this buffer was used to obtain the first peak (IgG fraction). The IgM-containing fraction was eluted with 0.1 M buffer, pH 8.1. The globulin fractions were concentrated to one-half of the original volume with polyethylene glycol (Carbowax) at 5°C. Each globulin fraction was determined by immunoelectrophoresis. The concentrated fractions were dialyzed against 0.01 M sodium phosphate buffer, pH 7.4, containing 0.15 M NaCl, and used for serologic tests. The IgG and IgM fractions were each treated with an equal volume of 0.2 M 2-mercaptoethanol in phosphate saline buffer, pH 7.4, at 37°C for 2 hours. The mercaptan was removed by dialysis against phosphate-buffered saline.

Results. The anti-B agglutinins, as well as heteroagglutinins against rabbit red cells, of pre-immune group O and group A sera were removed either by a single absorption or by repeated absorptions with rabbit red blood

TABLE I. Isoagglutinin Titers of Pre- and Post-Immunization Sera Before and After Absorption with Rabbit Red Cells.

		Saline method								Ficin method							
		Anti-A				Anti-B				Anti-A				Anti-B			
		Unabsorbed		Absorbed		Unabsorbed		Absorbed		Unabsorbed		Absorbed		Unabsorbed		Absorbed	
		Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post	Pre	Post
Group O*	1	128	512	64	256	128	256	0	0	256	1024	128	512	256	256	0	0
	2	128	1024	32	512	64	512	0	2	128	4096	64	2048	128	512	0	8
	3	64	128	32	32	64	128	0	0	128	512	64	256	128	256	0	0
	4	64	256	32	64	32	128	0	0	64	1024	32	512	64	512	0	0
	5	32	256	16	128	64	128	0	0	64	256	16	128	64	256	0	0
	6	32	256	16	128	16	128	0	0	32	512	16	128	32	256	0	0
Avg‖		64	256	32	128	64	256	0	0	128	1024	32	512	128	256	0	0
Group O†	7	128	512	64	256	64	1024	0	32	128	512	64	256	128	2048	0	64
	8	64	128	32	64	128	512	0	16	128	256	32	64	256	1024	0	64
	9	64	512	32	128	32	2048	0	32	64	1024	32	512	128	4096	0	64
	10	32	256	16	64	32	1024	0	16	64	256	32	128	64	2048	0	32
	11	32	256	16	128	16	256	0	8	32	1024	16	256	32	1024	0	32
	12	32	128	8	32	16	256	0	4	32	256	16	128	32	512	0	16
Avg		64	256	32	128	32	512	0	16	64	512	32	256	64	2048	0	64
Group A†	13	0	0	0	0	128	256	0	4	0	0	0	0	128	512	0	16
	14	0	0	0	0	64	256	0	8	0	0	0	0	128	512	0	8
	15	0	0	0	0	64	512	0	8	0	0	0	0	64	1024	0	32
	16	0	0	0	0	32	256	0	4	0	0	0	0	64	512	0	16
	17	0	0	0	0	32	128	0	4	0	0	0	0	32	128	0	8
	18	0	0	0	0	16	128	0	4	0	0	0	0	32	256	0	16
Avg		0	0	0	0	64	256	0	4	0	0	0	0	64	512	0	16
Group B*	19	128	512	128	512	0	0	0	0	128	512	128	512	0	0	0	0
	20	64	256	64	256	0	0	0	0	128	512	128	512	0	0	0	0
	21	64	256	64	256	0	0	0	0	64	256	64	256	0	0	0	0
	22	64	512	64	512	0	0	0	0	64	1024	64	1024	0	0	0	0
	23	32	128	32	128	0	0	0	0	32	512	32	512	0	0	0	0
	24	16	256	16	256	0	0	0	0	16	256	16	256	0	0	0	0
Avg		64	256	64	256	0	0	0	0	64	512	64	512	0	0	0	0
Group O‡	1		1024		512		256		2		4098		1024		512		8
	2		512		128		128		0		1024		512		256		0
Group O§	3		512		128		2048		16		512		128		4096		64
	4		256		128		1024		8		512		128		2048		32

* Individuals immunized with soluble blood group substance A. † Individuals immunized with soluble blood group substance B. ‡ Serum from mother of group A infant with hemolytic disease. § Serum from mother of group B infant with hemolytic disease. ‖ Avg values are rounded off to nearest whole number.

TABLE II. Demonstration of Cross-Reacting Antibody in Group O Serum.

Eluate	From	Reaction with A cells Saline	A cells Ficin	B cells Saline	B cells Ficin
I	Rabbit red cells	++	+++	++	+++
II < A	Human group A red cells	+	++	0	0
II < B	Human group B red cells	0	0	0	0

cells (Table I). Tests of the post-immune group O and group A sera produced by immunization with B substance showed that isoagglutinin activity remained even after 2 absorptions with rabbit red cells. The agglutinin titers were more enhanced as demonstrated by the ficin and indirect antiglobulin tests than by direct saline agglutination. Also shown in Table I, the anti-B agglutinins of immune group O produced by immunization with A substance were eliminated by absorptions (with the exception of one case). Of interest in the detection of immune type of anti-B antibody is that one of each of the group O sera obtained from the subject who was immunized with A substance, and from the mother who had group A infant with hemolytic disease, also showed agglutination of B red cells in the direct saline and ficin tests after absorption with rabbit red cells (no reaction with rabbit red cells was observed in the absorbed sera). The results also indicate that the immune group O sera might contain higher titers of cross-reacting antibody. Absorption did not reduce the anti-A titer of group B, and caused only a 1 to 2 tube reduction of anti-A of group O sera. The difference in the anti-A titer between group O and group B sera after absorption also seems to be due to the presence or absence of a cross-reacting antibody which exists in group O sera.

Repeated absorption (4 times) of the immune group O sera containing immune anti-B reduced the titer 1 to 2 tubes for anti-B, but the agglutinating activity was not abolished by multi-absorption.

In regard to the cross-reacting antibody, the eluates (Eluate I in Fig. 1) from the rabbit red cells after absorption of some preimmune and all post-immune group O sera were capable of agglutinating A and B cells; the results indicate the presence of cross-reacting antibody (Table II). However, the eluates (Eluate II in Fig. 1) from group B red cells sensitized with group O sera which had been previously absorbed with rabbit red cells, failed to produce agglutination of group A and B red cells. The positive reaction of group A red cells was observed in the eluate from group A red cells and no agglutination occurred with group B red cells.

In the presence of complement, human group B red cells were hemolyzed by the immune sera which contained anti-B antibody activity after absorption with rabbit red cells. None of the absorbed sera from preimmune individuals produced hemolysis of the group B red cells.

According to the present results, the rabbit red cells seem to have absorbed "naturally occurring" anti-B antibody leaving "immune" anti-B in the immune group O and group A sera. The blood groups of the rabbit red cells in this study according to the classification of Dr. Carl Cohen were not correlated with their specific absorptive property, and the red cells of most rabbits less than 3 months old failed to absorb completely the "naturally occurring" anti-B antibody and heteroagglutinins from post-immune group O sera even after 3 absorptions. Some of the adult rabbit red cells showed this specificity in absorption, but the serologic characteristics of the specific antigenic determinant in the rabbit blood groups were not distinguished in this study.

The immune anti-B antibody activity was demonstrated by the ficin method and indirect antiglobulin test in the IgG fraction only. In addition, the IgG fraction of most immune sera produced agglutination of group B red cells by direct saline method, but the reaction was weaker than the ficin and indirect antiglobulin tests. The titers of mercaptoethanol-treated IgG fractions were the same as untreated fractions. Hemolytic ac-

tivity was also found in the IgG fraction before and after treatment with sulfhydryl reagent.

Discussion. The present studies show that certain rabbit red cells, tentatively called B^R, react specifically with "naturally occurring" anti-B and leave in the serum the "immune" anti-B antibody. Friedenreich *et al*(11) subdivided the B antigen on erythrocytes and postulated that human group B red cells consist of 3 factors called B_I, B_{II} and B_{III}. Partial antigenic determinants B_{II} and B_{III} were found in rabbit red cells, and only B_{III} occurs in guinea pig red cells. They also observed that the anti-B in certain human sera could be absorbed with rabbit red cells. According to their concepts, the present results suggest that the "naturally occurring" anti-B agglutinin may consist of anti-B_{II} and anti-B_{III} antibodies which may be absorbed by rabbit red cells. In fact, Wiener(12) postulated that the "naturally occurring" isoagglutinins in man are produced by environmental factors, such as, exposure to microorganisms or other antigenic substances possessing human-like ABO antigens distributed in nature. The serologic characteristics of the "naturally occurring" isoagglutinins are, therefore, specific for the antigens which contain part of the antigenic structure in these substances. However, "immune" anti-B serum may contain anti-B_I in addition to the anti-B_{II} and anti-B_{III}, and the specific anti-B_I antibody may have an immune property which can be produced by stimulation with B antigen containing the B_I antigenic component. As mentioned above, human group B red cells contain more complex B antigen structure than animal red cells which may not possess the antigenic determinants similar to human group B red cells. However, it is possible that the soluble blood group specific substance B prepared from horse stomach may be antigenically related to B antigen of human red cells.

In regard to Witebsky's partial neutralization test(13), "immune serum" produced agglutination of group A_1 or group B red cells in the presence of group AB serum and by indirect antiglobulin test after neutralization of the serum with AB substance, while no reaction was observed in normal serum. However, an excess of A or B substance added to the "immune serum" abolished anti-A or anti-B agglutinin activity in the presence of AB serum and by the indirect antiglobulin test. Therefore, addition of a sufficient amount of blood group substance is required for this test in detection of immune anti-A and anti-B antibodies according to Witebsky's definition. This immune characteristic possessing a resistance to neutralization against A or B substance indicates that the results are due to a quantitative difference of immune type of antibody in the serum rather than a qualitative difference in relation to the A- or B-like antigens. However, the present method demonstrated that the presence of immune anti-B antibody was not abolished by the repeated absorption with rabbit red cells. Since the results also suggested that "immune serum" contains anti-B_I antibody with a different titer depending on the response of individuals to the injection of B substance, the B substance therefore seems to contain the B_I antigen.

The immune property of anti-B antibody in this experiment was found to exist only in the IgG fraction, and the fraction produced agglutination of human group B red cell by the direct saline, ficin, and indirect antiglobulin tests. Hemolytic activity of anti-B was also found in this fraction.

The B^R test may be a precise test system for detection of immune anti-B specific antibody, tentatively called anti-B_I, and the test procedure may be used for pre- and post-natal diagnosis of hemolytic disease of the newborn due to group B incompatible pregnancies.

Summary. Immune anti-B antibody was detected by absorption of sera with certain rabbit red cells (B^R), and agglutination and hemolysis of the absorbed serum with human group B red cells. The antibody (anti-B_I) activity was found only in IgG fraction. The method might be useful for detection of immune anti-B antibody in human sera especially in cases of hemolytic disease of the newborn due to group B incompatibility.

1. Winstanley, D. P., Konugres, A., Coombs, R. R. A., Brit. J. Haemat., 1957, v3, 341.

2. Konugres, A., Coombs, R. R. A., ibid., 1958, v4, 261.

3. Tovey, G. H., Lockyer, J. W., Blades, A. N., Flarell, H. C. G., ibid., 1963, v8, 251.
4. Yokoyama, M., Fong, S., Fudenberg, H. H., Fed. Proc., 1963, v22, 381.
5. Polley, M. J., Adinolfi, M., Mollison, P. L., Vox Sang., 1963, v8, 385.
6. Yokoyama, M., Fudenberg, H. H., J. Immunol., 1964, v92, 413.
7. ———, ibid., 1964, v92, 966.
8. Haber, G., Rosenfield, R. E., Andresen, P. H., Festskrift, Munksgaard, Copenhagen, 1957, p45.
9. Landsteiner, K., Miller, C. P., J. Exp. Med., 1925, v42, 853.
10. Levy, H. B., Sober, H. A., Proc. Soc. Exp. Biol. and Med., 1960, v103, 250.
11. Friedenreich, V., With, S., Z. Immunforsch., 1933, v78, 152.
12. Wiener, A. S., J. Immunol., 1951, v66, 287.
13. Witebsky, E., Blood, 1948, v3, 66.

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Anti-Inflammatory Activity of Nonsteroidal Agents in a Rat Granuloma Assay. (30320)

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The cotton pellet anti-inflammatory bioassay, although lacking in precision as an ideal procedure, has served as a useful tool to find therapeutically useful anti-inflammatory steroids. This method has been applied to evaluation of nonsteroidal compounds as anti-inflammatory agents and the results reported here indicate significant anti-inflammatory activity for 6 nonsteroidal compounds.

Methods and materials. Twenty-three- to twenty-five-day-old albino rats of the Charles River strain were bilaterally adrenalectomized under ether anesthesia. At the time of surgery 2 cotton balls, each weighing 5 ± 1 mg, were inserted subcutaneously. From the time of surgery the operated animals were fed a commercial complete diet in the form of a Purina chow and 0.9% saline was substituted for drinking water. The test compounds were dispersed in a vehicle consisting of sodium chloride (0.9%), polysorbate 80 (0.4%), carboxymethylcellulose (0.5%) and benzyl alcohol (0.9%) so that the total dose was contained in 0.2 ml. One day and 2 days after surgery the test animals received subcutaneous injections of 0.1 ml of the suspensions. Rats of the control groups received a total of 0.2 ml of the vehicle only. Twenty-four hours after the last injection, the rats were sacrificed with ether, the granuloma surrounding the cotton pellets was removed, the body weights were determined. The dissected granulomas were dried for 24 hours at 100°C and the dry granuloma weight was obtained by subtracting the weight of the cotton pellet from the gross weight. In most trials each of the 2 granulomas in a given animal were considered to be individual observations. In some experiments the sum of the 2 granuloma weights was treated as a single observation.

In most of the experiments the test material was administered by subcutaneous injection. In one trial the test compound and standard were incorporated into the diet and the dose expressed as mg of compound per kilogram of food. In these studies the experimental diet was fed for 48 hours.

Results and conclusions. Tables I through VIII summarize the anti-inflammatory activity of 6 compounds studied by the subcu-

TABLE I. Anti-Inflammatory Activity of Subcutaneously Administered Chlorpheniramine Maleate (CM).

Material administered	Total dose, mg	No. of observations	Mean body wt, g	Mean dry tissue wt, mg ± S.E.
O	0	20	57	16.5 ± 1.1
Cortisol acetate	.4	24	57	14.9 ± 1.1
	.8	20	57	11.9 ± 1.2
	1.6	21	55	10.8 ± .8
CM	1	21	53	15.7 ± 1.5
	5	20	52	11.4 ± 1.4

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