

DISTRIBUTION OF "BLOCKING" ANTIBODY IN HUMAN SERUM PROTEINS FRACTIONATED BY ELECTROPHORESIS-CONVECTION¹

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Campbell, Cann *et al.* (1) were the first to apply the gentle separatory method of electrophoresis-convection (EC) to the study of antibody distribution in the proteins of human serum. After exposing allergic serum to two stages of EC as well as to a final salting-out procedure for fractionation, and analyzing the yields for electrophoretic composition as well as for sensitizing qualities in normal skin, they concluded provisionally that the reagins of their two specimens did not occur predominantly in γ -globulin but were probably restricted to the α - and/or β -globulins. Increasing the separatory phase to 8 stages of EC and using more extensive immunologic tests for activity, Loveless and Cann (2, 3, 4) have examined 5 sera to conclude that the β -globulins play a dominant role in sensitization.

Similar exploratory studies were then undertaken for the first time on the blocking antibody, which coexisted with reagins for the same pollen antigen in one specimen. When comparison was made between the sensitizing and the blocking powers of successive fractions, differences were noted. In fact, the sensitizing antibody appeared to depend upon the amount of β -globulin present in the various preparations whereas blocking activity followed γ -globulin content more closely, as has been briefly reported (2)².

The purpose of the present article is to give a detailed account of the work on this pollen-

reactive, blocking serum and to present data on two additional blocking sera obtained from a diabetic woman who had acquired a marked refractoriness to insulin.

MATERIAL AND METHODS

Source of antiserum for insulin. B. E. R., a woman in her middle sixties, had controlled her diabetes with conventional doses of protamine zinc insulin since 1942. During 1951 her daily requirement gradually rose from 35 to 120 units. In December of that year, 1000 units had to be given during the first 24 hours of hospitalization in order to overcome a mild state of acidosis. Thereafter her dosage was adjusted according to several daily tests for urinary acetone and sugar. From 140 to 300 units of crystalline zinc insulin sufficed until August 1952, when she was again hospitalized for acidosis. Management at this time was complicated by the patient's tendency to develop occasional hypoglycemia. Although she remained in the hospital for 11 months under strict dietary control, it proved impossible to control her diabetes with fewer than from 900 to 1100 units daily. ACTH therapy only increased the glycosuria and led to acidosis; hence it was discontinued after five days.

It was assumed that B. E. R. possessed circulating antibodies for insulin. This was based on the observation of Lowell (6) that insulin refractoriness in man is associated with a serum-borne factor which interferes with the hypoglycemic effect of this hormone on mice; and on our experimental development of heat-stable, blocking antibody (7) in a diabetic who received graduated

a starch column after zone electrophoresis. Whereas the precipitating antibodies migrated largely in association with a slow-moving component, the sensitizing bodies moved in general more rapidly. These findings are not inconsistent with our own, as will be shown.

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² Since this article was submitted for publication, Kuhns (5) has shown that skin-sensitizing antitoxin may be differentiated from the precipitating type of antitoxin in human serum by virtue of their different, albeit overlapping, locations on

doses of insulin to overcome her allergy toward insulin (8). Preliminary experiments in September, 1952, substantiated this assumption for B. E. R. in that mouse-protective as well as blocking anti-allergenic qualities were demonstrated in her serum. In order to procure sufficient serum to permit fractionation by electrophoresis-convection, a pint of blood was taken on October 23, 1952. Food had been withheld overnight and no hormone was given after a dose of 350 units of commercial crystalline zinc (pork and beef) insulin had been administered 24 hours before venipuncture. Inasmuch as the blood was found to contain 500 mg % of sugar, it can be assumed that this antigen had left the circulation by the time the blood was drawn.

Within several weeks the patient's need for insulin rose gradually to between 2000 and 3000 units daily, as much as 3390 units being required on one occasion in middle November.

Meantime, she developed an unexplained, low-grade fever, associated with an increased white cell count amounting to 29,000 per cu mm of blood, 75% of the cells being eosinophils. Biopsies revealed many eosinophils in the lymph glands and bone marrow. In spite of the fact that at times she developed a 10-mm wheal at the site of her insulin injection, endermal tests with .025 ml of 40-unit pork and beef crystalline zinc insulin elicited no reaction. On the theory that her occasional mild attacks of dyspnea might be referable to the type of insulin being used, all-beef insulin was commenced in late 1952 and has since been employed.

During mid-January, 1953, the patient developed acute fever, dyspnea and pulmonary infiltration which lasted two weeks and were believed to be of viral origin. Procaine penicillin and dihydrostreptomycin were given in full doses. During this period a dramatic reduction occurred in her leukocytosis, eosinophilia and apparent insulin refractoriness, daily doses of 300 to 750 units of insulin now controlling her diabetes. Indeed, on several days she was given no insulin at all because of recurrent hypoglycemic symptoms. Forty-eight hours after one such episode, which was characterized by a blood sugar level of only 30 mg % and convulsive movements, the February 3 sample of blood was taken for fractionation. This venipuncture was done 12 hours after 150 units of insulin and a meal had been given.

Although no subsequent samples have been taken for fractionation, the patient's clinical progress will be briefly recounted because of its immunologic implications. The insulin requirement

rose gradually to 1400 units by February 24, 1953 (4000 units having been required during one 36-hour period when intravenous fructose was being given experimentally), while her clinical state fluctuated between acidosis and hypoglycemia without predictability. Eosinophilia, leukocytosis and elevated temperature recurred. On March 3, 1953, oral cortisone therapy was instituted on an empirical basis. Within the first week it was possible to institute a gradual reduction of the insulin intake.

By the last week in March, the daily dose had become stabilized at between 300 and 525 units in conjunction with 75 to 100 mg of cortisone. When an attempt was made in June to omit the cortisone, she developed within a week wheezing, elevation of temperature to 39°C, acidosis and the subsequent need for 2100 units of insulin daily. Cortisone therapy was therefore reinstituted and has been maintained to date. Following this reinstitution, it was possible to dismiss the patient from the hospital (on July 8, nearly a year after her admission) on a regimen of 500 units of insulin and 100 mg cortisone. Aside from two brief hospitalizations for subcutaneous infections, she has remained ambulatory and is at the time of this writing stabilized on about 120 units of insulin daily along with 75 mg of cortisone. Her output of eosinophils and white blood cells is in the vicinity of normal and she is experiencing little or no difficulty which might be attributed to allergic reactions in either skin or lungs.

Source of antiserum for pollen. K. U. was a middle-aged man who had a rather severe form of typical hay fever due to ragweed pollen. Three weeks prior to venipuncture he had completed an intensive course of immunization comprised of nine weekly intracutaneous injections of ragweed extract which involved a total dose of 313 μ g of phosphotungstic-acid-precipitable N (31,300 PtaN or so-called protein N units). Unlike the two specimens procured from the insulin-refractory subject, this serum contained, in addition to its blocking factor, a plentiful amount of sensitizing antibodies for the antigen in question.

Fractionation of serum was effected by successive stages of EC in the cold at pH levels which were graduated stepwise from 8.1 to 5.2, as previously described (2, 3).

At the conclusion of each 48- to 72-hour experiment, the contents of the upper reservoir of the EC apparatus were removed, dialyzed against phosphate buffer at pH 7, passed through a bacteria-retaining filter, labelled "Top Fraction I"

for the first stage, "Top II" for the second, etc.; and stored at 4°C. The portion remaining in the apparatus (Bottom Fraction) was now equilibrated by dialysis against the buffer to be employed for the next fractionation, diluted with buffer to compensate for its loss of Top Fraction, and then subjected to EC. Bottom Fraction taken from the 8th stage was mixed with an equal volume of saturated ammonium sulphate at pH 7 so as to provide fractions BG (bottom globulin) and BA (bottom albumin).

All fractions were assayed for N content by Kjeldahl digestion and Nesslerization, and were analyzed for electrophoretic composition in the apparatus of Perkin and Elmer, barbital buffer being used at pH 8.1 in 0.1 ionic strength. The mobility and the relative concentration of each component in a given preparation were calculated from enlarged electrophoretic diagrams according to Pedersen (9).

Antigens. In order to minimize technical variables in our activity tests, one uniform source of crystalline zinc insulin (Squibb pork and beef) of 500-unit strength and one glycerin-stabilized stock of pollen extract containing 70,000 protein N units per ml were used for the preparation of serial, twofold dilutions every few weeks. Isotonic saline solution served as the diluent. In the case of insulin, the saline was fortified with 0.25% heated human albumin and 1:10,000 merthiolate.

Preliminary tests for reagin and for blocking antibody. When qualitative Prausnitz-Kustner tests were carried out in normal skin with 0.1 ml of each of the three sera to be fractionated, 24-hour challenge tests with the related antigen elicited only borderline responses in B. E. R.-prepared sites whereas they provoked strongly positive wheal and flare reactions in K. U.-injected areas. Although additional sensitization studies were subsequently done with serial dilutions of the K. U. serum and with K. U. serum:antigen mixtures, these results with the thermolabile antibody have been published elsewhere (3) and will not be repeated here. They had little bearing on the present investigation beyond showing that the course of pollen injections given K. U. induced him to form not only blocking factor but an increased amount of sensitizing antibodies as well. The blocking antibody production was appraised by adding *heated* K. U. serum to a known reagin:allergen system, and it was found that adequate block was present to

raise the neutralization requirement eight-fold. K. U.'s conjunctival tolerance was also found elevated by a factor of 10 after the immunizing course of injections, reflecting the acquisition of this antibody by the tissue.

Both B. E. R. sera, when tested in heated form for their effect on the neutralization requirement of a known, insulin-allergic serum, proved to possess blocking antibody. *Fractions* prepared by EC were, however, difficult to titrate reliably due to their lowered protein and antibody concentrations. It was therefore necessary to resort to a type of direct blocking test which had been described in 1940 (7) but which has since received little attention.

Direct blocking test for thermostable antibody. In this test, the presence of excess antigen in a serum:antigen mixture is revealed by its capacity to provoke a whealing response in sensitized human skin. If blocking antibody is present in the serum, more antigen must be included in the tube than when control, normal serum is mixed with the allergen. The difference expresses the allergen-binding power of the immune specimen in terms of units blocked per ml of serum.

In order to compare the blocking qualities of different derivatives of B. E. R. serum, replicate areas on the dorsal surface of the trunk of a normal subject were intracutaneously prepared with 0.1 ml of 1:3 insulin-sensitizing serum. The sites were so located as to form a Latin square containing about six or eight columns and six to eight rows. In 24 hours, each dye-marked area received .04 ml of a mixture containing an equal volume of fraction and of graded antigen, a 0.25-ml syringe and gauge-26 needle being employed for the endermal injection. The assignment of mixtures to sites was randomized in order to minimize the between-columns and between-rows error. Antigen strengths were so selected as to provide wheals ranging from 1 cm in cross diameter to negative (recorded as + + +, + +, +, ± or 0). The ± or minimal reaction was chosen as the uniform end-point because we, like Swain and Becker (10), find that wheal diameter is not a sensitive means of evaluating reagin:allergen responses in man. The strength of antigen required for the threshold response in the presence of different fractions could then be compared, thereby indicating their relative contents of blocking antibody per ml. Since the blocking values thus obtained were found to vary with the

normal subject employed for the titration, it was important to examine all the derivatives of a selected serum in the same experiment. In order to utilize each sensitized area most effectively, therefore, it was our practice to inject a trial allergen:fraction mixture for each derivative at the outset of the 24-hour experiment. Depending on the size of the resulting wheal, a second site was then challenged with a mixture calculated to evoke a response at the opposite end of the reaction scale. Thereafter, it was relatively simple to procure the desired intermediate responses by employing intervening strengths of antigen in a few additional mixtures.

Since the blocking power appeared to be the same regardless of whether the fractions had been heated at 60°C for two hours or tested in their original state, this variable will in general be disregarded in presenting the findings. The same situation obtains with reference to incubation of allergen:antibody mixtures, no difference in blocking quality being noted whether mixtures were used immediately or were allowed to stand at either 4° or 28°C for one to 24 hours.

Test subjects were nonallergic, healthy men in their third decade whose skins gave no irritative responses to the sera to be titrated or to the highest concentrations of allergen to be employed in the study. They were readily sensitized with the standard reaginic sera to be used in 1:3 dilution. As many as 11 different subjects participated in one or more blocking experiments on a given set of serum derivatives.

Handling of titration results. The average blocking power of a selected fraction was first determined by examining the several titrations which had been carried out on an individual test subject. The representative result was then grouped with similar data from other subjects so that a median value could be assigned the fraction. In order to adjust for differences in the protein content of the several fractions, this blocking activity per ml was now converted to blocking power per mg of protein, by the simple device of dividing the median number of units blocked by the assay figure for protein in the fraction. This antibody activity per mg protein will be called an "activity ratio." The use of such ratios involves the presently necessary assumption that activity in the various fractions is due to the same substance or group of substances and is not influenced by the total protein content *per se*. This procedure, therefore, within the

validity of the assumption, gives an indication of the relative potencies of the several fractions and permits correlation of activity with the electrophoretic composition of the various preparations. In making these comparisons, the limit of reliability of the titration method has been estimated to be one half dilution tube of antigen, as one or two observations on each graph will indicate.

Mouse protective tests of a type employed by Lowell (6) were done with weanling and young adult mice which had been fasted overnight and which were maintained at 37°C following their injection with mixtures of insulin and well dialyzed serum. Comparison was made between insulin-resistant and normal serum both as to the time of onset of symptoms (convulsions, paralysis) and the incidence of death, when amounts of insulin ranging from .02 to .075 unit were injected along with .35 ml to .5 ml of whole serum or 1 ml of fraction. From 15 to 63 mice were involved in each experiment.

RESULTS

Preliminary mouse-protective tests. Amounts of insulin which killed 94% of a group of 30 mice given control serum were lethal for 64% of the 42 animals given B. E. R. immune serum. Furthermore, the latter lived longer than the control group, the median survival time ranging from 78 minutes to five hours, depending on the experiment, whereas the corresponding figure for the controls was 45 to 90 minutes. In two additional experiments on 30 mice, an equal proportion of each group succumbed (87%), so that only the survival time could be used as an index to antibody effect. The median animal in the two control sets died at 68 and 120 minutes, respectively, whereas 127 and 177 minutes elapsed for the comparable members of the B. E. R. group. Although these results suggested a difference between normal and insulin-resistant sera, the degree of protection was not as striking as that demonstrated by Lowell (6) or by us (4) for three other refractory specimens.

When repeated attempts were made to determine the relative mouse-protective qualities of *derivatives* of our B. E. R. sera, this type of test proved unfruitful. In retrospect, our failure to discriminate among fractions may have stemmed from the rather low order of protection afforded mice by the unfractionated specimens; from the low antibody content per ml of most of

TABLE I
Direct blocking tests carried out with fractions heated 2 hours at 60°C

Fraction	B. E. R. 10/23/52 Median for 11 Subjects		B. E. R. 2/3/53 Median for 2 Subjects		K. U. 8/14/52 Median for 3 Subjects	
	Insulin	Insulin	Insulin	Insulin	Ragweed	Ragweed
	Units blocked per ml*	Units blocked per mg protein	Units blocked per ml†	Units blocked per mg protein	Units blocked per ml†	Units blocked per mg protein
(Normal serum)	(1:20).03		(1:20).3 (1:10).6		(1:20 or undil.) 1.25, 20, 7.5 (undil.) 300	
Whole serum	(1:20).312	.085	(1:10)2.4	0.30		4.3
Top I undiluted	.312	.135	3.7	0.91	50	14.3
Top II undiluted	.0011	.0004	4.13	0.64	10	5.0
Top III undiluted	.007	.003	4.0	0.97	10	7.7
Top IV undiluted	.001	.0004	4.0	1.17	12	12.0
Top Va undiluted	.312	.115	2.1	0.58	53	40.7
Top Vb undiluted	.25	.167	1.47	0.58	15	31.2
Top VI undiluted	.625	.284	1.59	0.37	15	11.5
Top VII undiluted	.0006	.00016	1.15	0.18	13	4.1
BG-7 undiluted	.0045	.00054	1.46	0.13	5	3.9
BA-7 undiluted	.0001	.00001	0.62	0.0036	1	0.1

* No correction was made for the normal serum:insulin end point.
† Correction has been made for the normal serum:insulin end point.

the derivatives which could not be adequately compensated by increasing the volume for so small an animal; and from the limited number of animals that could be observed during a given experiment, making it possible to compare only three or four fractions at a time. These shortcomings were obviated by utilizing the direct, blocking procedure. It was provisionally assumed that the antibody which interfered with the reagin:insulin whealing reaction was the same as the one which acted as an antihormone for the patient and for the mouse.

Direct blocking tests on B. E. R. 10/23/52 insulin-resistant serum. The activity ratios (units of insulin blocked per mg total protein) for the EC derivatives of this serum are listed in Table I. They are based on end-points determined during 26 comprehensive experiments on 11 normal subjects (confirmed at a much lower level in two other individuals). No correction has been made in this set of results for the activity of insulin in the presence of normal serum, because the use of this baseline provided negative figures for certain of the preparations of low activity, which were awkward to handle.

Blocking activity will be noted in Table I and Figure 1 to be greatest in Top Fractions I, Va, Vb and VI. In view of the limit of reliability of the titration method (indicated for two observa-

tions on the graph), these four products may be considered equal in potency. Since Top I was isolated at pH 8.1 and Tops Va, Vb and VI were prepared by EC at pH 6 and 5.5, it is obvious

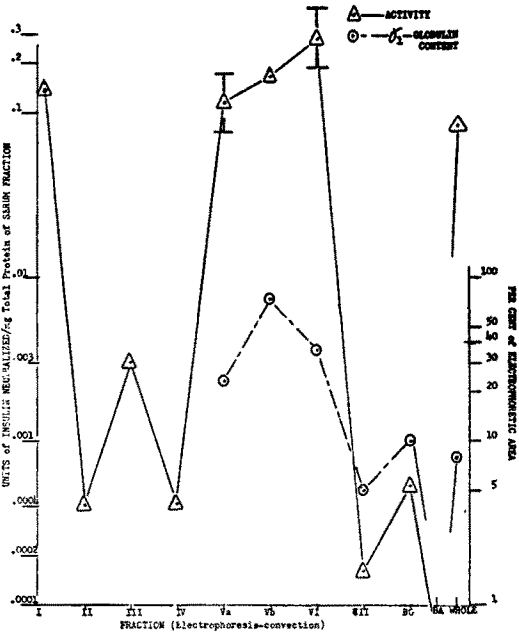


Figure 1. Blocking activity of insulin-resistant serum, B. E. R., 10/23/52 (median values based on 26 experiments in 11 test subjects).

TABLE II

Electrophoretic composition of 3 "blocking" sera examined at pH 8.1 in barbital buffer, ionic strength 0.1, field strength 7 v/cm

Fraction	pH of EC Run	Total Protein Content	Distribution of Components in Fraction (Per cent of Total Area)					
			Albumin	α_1 -Globulin	α_2 -Globulin	β -Globulin	γ_1 -Globulin	γ_2 -Globulin (mobility -2×10^{-6} or greater)
Whole B.E.R., 10/23/52.....		mg/ml						
Top I.....	8.1	74.0	40		4.0	24	8	24
II.....	7.5	2.3	2			2		96
III.....	7.0	2.7	4			5		91
		2.3	4			4		92
Top IV.....	6.5	2.4	3		3	5		89
Va.....	6.0	2.7	5				23	72
			3*		1*	3*	15*	78*
Vb.....	6.0	1.5	2		6	19	73	
Top VI.....	5.5	2.2	4	4	26	30	36	
VII.....	5.2	3.5	14	42		39	5	
BG-7.....	7.0	8.3	5	17	68		10†	
BA-7.....	7.0	6.6	92		8‡			
Whole B.E.R., 2/3/53.....		80.0	32	8	15	20	9	16
Top I.....	8.1	3.4	1		3	4		92
II.....	7.5	5.5	8	3	5	6		78
III.....	7.0	3.5	9	3	5	6		77
Top IV.....	6.5	2.9	6	5	5	8		76
Va.....	6.0	2.6	3		5	17	75	
Vb.....	6.0	1.5	5		12	17	66	
Top VI.....	5.5	2.7	11		28	33	28	
VII.....	5.5	3.1	24	26	13	30	7	
BG-7.....	7.0	6.7	7	17	70		6†	
BA-7.....	7.0	5.5	77	13	7	3		
Whole K.U., 8/14/52.....		70.0	56		5	24§		15
Top I.....	8.1	3.5	3			6		91
II.....	7.5	2.0	5			2		93
III.....	7.0	1.2	6			4		90
Top IV.....	6.5	1.0	5			5		90
			5*			6*	39*	50*
Va.....	6.0	1.3	6			25	69	
			6*			20*	37*	37*
Vb.....	6.0	0.48	12			24	64	
Top VI.....	5.5	1.3	14		15¶	58	13	
VII.....	5.3	3.2	30		25	45		
BG-7.....	7.0	1.3	9	62	28	1†		
BA-7.....	7.0	9.0	89		11‡			

* Rising pattern.

† May be a mixture of β -globulin and γ_1 -globulin.

‡ Contains small amount of α_1 - and β -globulin.

§ Resolved into 2 well defined components in descending pattern, 3 in rising pattern.

¶ Contains a small amount of α_1 -globulin.

that molecules which could be immobilized at these H-ion concentrations were the ones chiefly responsible for blocking activity. Although it appears on Figure 1 that Fraction III carried a significant amount of blocking power, this is partly due to the use of logarithmic graph paper, the actual difference between Tops III and I amounting to 50-fold.

When the electrophoretic compositions of the various preparations were examined, γ -globulin was found to be the major constituent of the most active. Top I, for example, was comprised 96% of this component, while Va contained 95%, Vb 73%, and VI 36% (Table II and Fig. 2). Although the relatively inactive Fractions II, III and IV also were rich in γ -globulin, the latter constituent was unique in that it carried an intermediate charge whereas in the potent fractions the γ -globulins possessed either very low or very high charges. (Their average mobility rate in Top I was -1.06×10^{-5} cm² sec⁻¹ volt⁻¹ whereas the comparable figure for the Tops Va, Vb and VI ranged from -1.8 to -2.56×10^{-5} while the γ -globulins in intermediate fractions carried a rate of between -1.23 and -1.46×10^{-5} .) The per cent of fast moving, or γ_1 -globulin in successive fractions is plotted in Figure 1. The correlation with activity was found to be closer for this component than for any other constituent (except the γ_2 -globulin of very low mobility), when each was compared in this way with the activity curve.

Albumin could virtually be excluded from consideration as a carrier of blocking antibody in view of the negligible activity of preparation BA which was comprised 92% of albumin. The correlation was equally poor for the rest of the albumin-containing fractions, since their contents of this protein were similar and low whereas their potencies varied widely.

The α_1 - and α_2 -globulins appeared to be similarly unrelated to the blocking function, particularly in the case of BG which was 85% α -globulin and showed little insulin-binding capacity. Fraction VII was also rich in this globulin, containing 42% according to area measurements, yet it exhibited only minor blocking qualities. Conversely, Fractions Va and I carried major activity although they possessed no detectable α -globulin. In view of the small amount of α -globulin in Top Vb and the large proportion in Top VI, this protein was presum-

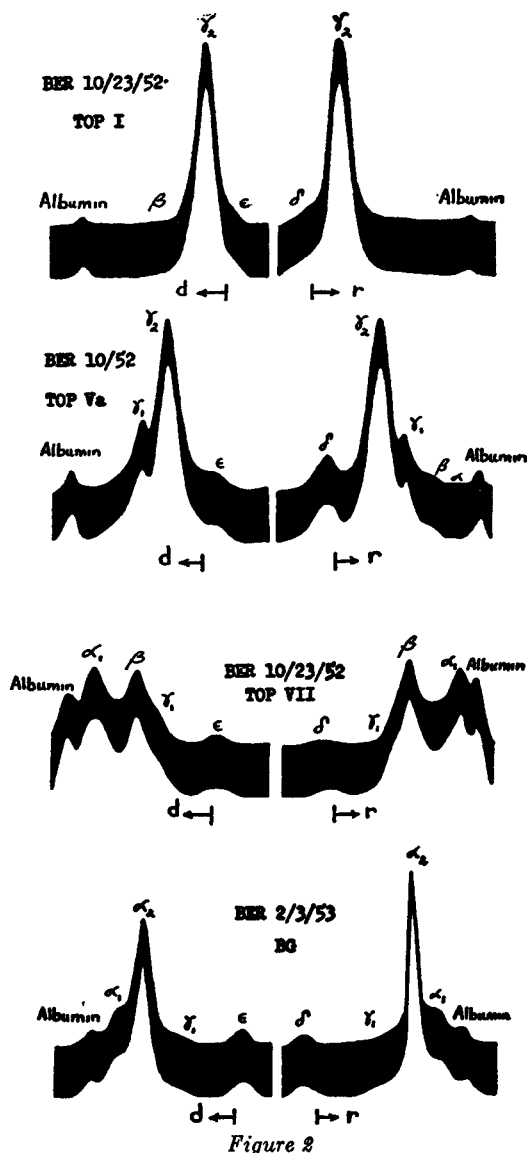


Figure 2

ably not responsible for their marked and comparable blocking powers, the latter being more logically attributed to their γ -globulins.

The β -globulins also failed to show a correlation with blocking activity, there being almost none of this component in the potent Top Va and only a trace in the highly active Fraction I. Conversely, Top VII which was richest of all in β -globulin carried only $\frac{1}{1000}$ as much block as the essentially β -free preparations, I and Va.

By elimination, therefore, as well as by the establishment of the positive correlation shown

above, certain γ -globulins seemed to be responsible for insulin-binding activity. At the time of the first bleeding, only the most slowly and the most rapidly mobile of the γ -globulin molecules appeared to be participating outstandingly, thus endowing the activity curve for the family of derivatives with a bimodal configuration. However, all the γ -globulins, regardless of charge, had acquired antibody activity by the time a second specimen of serum was procured 3½ months later.

The foregoing results indicate that EC successfully concentrated blocking antibodies in certain fractions. That this enrichment was achieved without damage to the immune bodies is implied by the finding that the activity ratio of VI was 3 times greater than the ratio for Whole Serum.

Fractions of B. E. R. 2/3/53 insulin-resistant serum. Figure 3 is based on five experiments in two normal subjects,³ median blocking values per ml having been corrected to the baseline for normal serum:insulin mixtures before being converted to activity per mg protein. The distribution of activity among the serum proteins had changed somewhat in the 3½-month interval between bleedings, Tops II, III, and IV having acquired maximal blocking qualities. In other words, not only those preparations which contained slow- and fast-moving γ -globulins, but

³ The antibody relationships among the various derivatives were confirmed in a third subject, N.E.L, but at a much lower level. A sensitized area in this normal man responded to as little as .019-unit insulin in the presence of 1:10 normal serum, whereas the corresponding end-point for the two other individuals lay in the vicinity of 0.6-unit insulin. This difference in the capacity of normal subjects to acquire local sensitivity is no doubt responsible for the apparent enhancement of antibody output in B. E. R. during the interval between venipunctures. As a matter of fact, when the two unfractionated sera were compared in DOR in March, 1953, the early specimen blocked 0.9-unit insulin whereas the other required 2-unit. These results were only slightly different from each other and approximated the end-point which had been obtained for the October sample in this recipient three months before. Thus it could be concluded that the blocking factor had been stable at 4°C for some months and that the patient maintained her output of immune bodies in spite of a temporarily reduced insulin requirement in February.

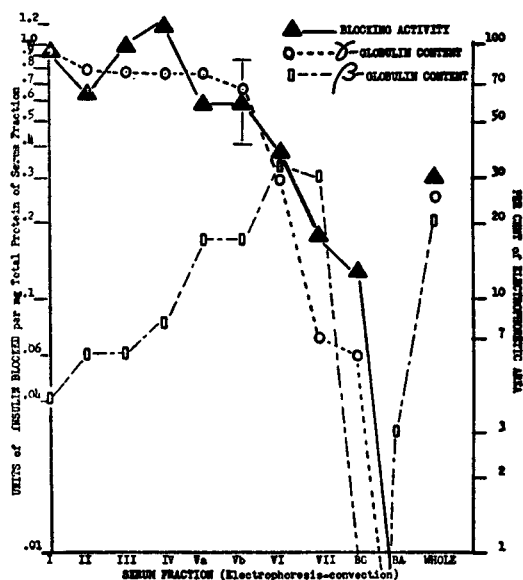


Figure 3. Blocking activity of insulin-resistant serum, B. E. R., 2/3/53 (median values from 5 experiments on test subjects D. O. R. and L. U. D.).

also those possessing γ -globulins of intermediate mobility rate, were now participating fully in the immune response. (As in the earlier fractionation, these mobility rates ranged from -0.8 to -2.6×10^{-5} .) Correlation between the blocking ratio and the total γ -globulin content of the several derivatives can be seen in Figure 3 to be positive and to contrast sharply with the situation for β -globulins.

When the area measurements of other constituents were compared in this way with the activity ratios of the related fractions, no correlation could be found. Albumin, for example, could virtually be excluded from consideration on the basis of the low activity of Fraction BA, which consisted 77% of albumin. Furthermore, other preparations which possessed little albumin exhibited strong activity. The α -globulins could be considered unimportant to activity because Fraction BG, which was comprised 87% of α -globulin, could block only $\frac{1}{10}$ as much insulin/mg as Top IV which possessed only 10% of this globulin. (See BG in Fig. 2.)

From these correlation studies, it became apparent that the only serum constituent to vary consistently with activity was γ -globulin. In this experiment, as in the earlier one, the separatory procedure of EC had concentrated antibodies in certain derivatives, increasing

their activity ratios by as much as fourfold over the starting material.

K. U. pollen-immune serum. Figure 4 shows the distribution of blocking factor among the heated fractions of K. U. serum (which carried not only thermostable blocking bodies but also heat-labile sensitizing antibodies, or reagins). The median values derived from seven direct blocking experiments on three normal subjects have been corrected for the control results obtained with pollen antigen and two normal sera in 1:20 dilution (Table I). The plotted ratios represent, therefore, the amount of precipitable, pollen N which could be neutralized by 1 mg of fraction protein. It will be seen that Tops Va and Vb, derived as usual from EC at pH 6, exhibited the greatest potency. Fractions I, IV and VI took second place. (Fraction I had originally shown the same maximal activity as Top Va when preliminary blocking tests were carried out with allergen:fraction mixtures in six naturally sensitive skins. Its later inferiority suggested a relative instability at 4°C.)

The activity curve for the 10 K. U. fractions resembled that of the early B. E. R. serum in its general configuration, although the differences among derivatives were less sharp, and Tops I and VI were relatively less potent. The electrophoretic findings also resembled those of the insulin-resistant specimens. Correlation studies set up between the activity of successive fractions and the area measurements of their individual

components again gave better evidence for the immunological role of γ -globulin than of other constituents. A minor exception was encountered in the case of Fraction BG, which contained no detectable γ -globulin. Its activity, however, was not great, amounting to only $\frac{1}{10}$ that of Va. If one postulated that its high concentration of α -globulin contributed the activity, it was necessary to conclude that α -blocking power per mg was of a very low order. In view of the complete lack of these globulins in the most effective preparations, Va and Vb, the concept of α -globulin activity was not appealing. On the other hand, the material designated as β -globulin in the electrophoretic pattern could well have been contaminated with a small amount of γ_1 -globulin, and this is the likely explanation for the observed activity of BG. The same statement applies to Top VII. The possibility that either albumin or β -globulin was associated with blocking qualities was dismissed for reasons already given (see B. E. R. analyses).

It was of interest to compare these activity ratios with others set up for the same fractions when their *reagins* were appraised in unheated form by means of the serum-dilution technique. As has already been reported for this and several other sera (3), the sensitizing activity reached a sharp peak in the region of Tops Va and Vb, resembling the blocking antibody curve in this respect. The reaginic ratios differed from the blocking ratios, however, in the case of several fractions. Top VI, for example, had maximal sensitizing qualities whereas it was only intermediate in effectiveness as a blocking agent. The contrast was even greater in the case of Top VII, which was almost as strongly sensitizing as the best fraction but was one of the least efficient blocking preparations. In the case of Top I, the situation was reversed in that the sensitizing power amounted to only $\frac{1}{15}$ that of the best preparation whereas in blocking tests the fraction carried over $\frac{1}{5}$ the antibody of the most powerful derivative. Whereas blocking activity among the K. U. fractions seemed to be more closely allied to the concentration of their γ -globulin, sensitizing power appeared to follow the β -globulin content.

Since there were appreciable differences in the distribution of the sensitizing and the blocking antibodies in K. U. serum, one is entitled to deduce that the two were distinct entities even

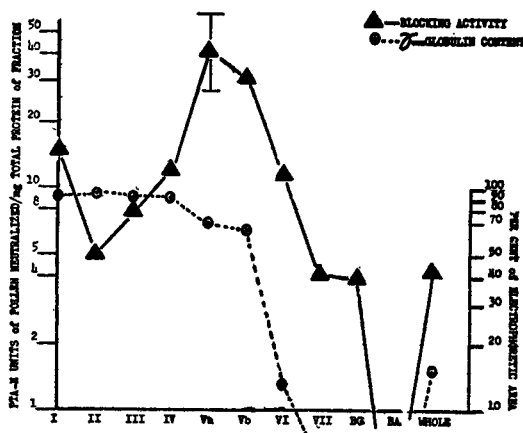


Figure 4. Blocking activity of ragweed-immune serum, K. U., 8/14/52 (median values based on experiments in subjects N. A. S., L. A. W., and R. E. I.).

when formed against a common antigen in the same individual.

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SUMMARY AND DISCUSSION

Sera procured from a pollen-immunized and from an insulin-refractory donor have been fractionated extensively by EC and examined for blocking activity as well as for electrophoretic composition. EC, which segregates molecules chiefly according to their electric charge, provided fractions which were comprised largely of proteins of low, intermediate or high mobility. One preparation, for example, consisted 96% of slow-moving or γ_2 -globulin, whereas another contained 75% of fast-moving γ_1 -globulin, and a third possessed 58% of β -globulin, while still another was comprised 90% of α -globulins. The remainder of the serum could be salted out so as to provide a final fraction which contained as much as 92% of albumin (see Table II). The foregoing components differed from each other in that they exhibited graduated mobility rates when examined at pH 8.1, the γ_2 -globulins migrating most slowly while the albumin moved most rapidly.

When such fractions were mixed *in vitro* with their respective allergens, a slight excess of the latter could be detected by testing the mixture in sensitized, normal skin. The amount that had to be added to one fraction to be slightly in excess of its blocking power could be compared with the amount required for other fractions, thus indicating their relative antibody content. When blocking power was expressed in terms of the total protein content of the preparation, it was found that activity of successive fractions fluctuated roughly with their γ -globulin concentration whereas no positive correlation with immunologic properties could be demonstrated for other serum constituents. In the first blood taken from the insulin-refractory donor, the γ -globulin of lowest (Top I) and highest mobility (Tops Va, Vb, and VI) were the chief participants in the blocking function, whereas all γ -globulins appeared to be similarly involved

in a later specimen. The activity curve of fractions prepared from the pollen-immune donor, K. U., resembled that of the early insulin-resistant sample.

The blocking activity of the 10 K. U. fractions did not duplicate relationships found among these preparations when their sensitizing antibodies were titrated by the technique of serum dilution (3). Although there was admittedly an area of overlap in the region of Tops Va and Vb, where maximal blocking and sensitizing activity coexisted, there was distinct blocking activity to the left of this area on the activity curve (notably in the Top Fraction, I), whereas important reaginic qualities were encountered to the right of this peak (Tops VI and VII). At the same time it was noted, in examining the electrophoretic data, that over 90% of the area of fractions to the left was made up of slow-moving γ_2 -globulin, while the β -globulin-enriched preparations lay in the central and right-hand regions of the curve (Tops Va, Vb, VI and VII). Fast-moving γ_1 -globulins, on the other hand, were concentrated largely in Tops Va and Vb and to a lesser extent in VI. This concentration of both the β -globulins and the γ_1 -globulins in Fractions Va, Vb and VI made it difficult, at first glance, to decide whether the two types of antibody were related to one or both of these serum proteins. Investigation of the area and activity data for the remaining fractions, however, made it clear that the β -globulins were concerned with sensitizing antibody whereas γ -globulins carried blocking qualities. It was not surprising, therefore, that fractions rich in both these components should also be efficient in both of these antibody functions.

As might have been expected from the fact that Fractions Va, Vb and VI had been immobilized and isolated at pH 6 and 5.5, the electrophoretic mobilities of their β -globulin and γ_1 -globulin molecules differed from each other only slightly, so that the complete separation of these two types of globulin poses a difficult technical problem. We have recently attempted to accomplish this by rerunning Fraction Vb of a reaginic serum at a slightly different pH level and were rewarded by the finding that sensitizing antibody and β -globulin content had both been concentrated in Top Vc (4). Similar efforts with blocking serum have not as yet been made.

The generally lower mobility of the blocking

antibody justifies the conclusion that it is an entity distinct from the sensitizing antibody, the latter being associated chiefly with molecules of greater mobility. That some overlapping of the 2 activities occurred in fractions of intermediate mobility, such as Tops Va and Vb, is not surprising. A similar situation was encountered by Kuhns when he subjected human antitoxic sera to zone electrophoresis on a starch column. Whereas the precipitating antibodies migrated in association with a slow-moving component, the skin-sensitizing bodies moved in general more rapidly, with some overlapping at the interface.

The clinical behavior of our insulin-resistant patient, as well as the outcome of mouse-protection tests, made it apparent that she possessed some means of interfering with the hypoglycemic effect of insulin. Blocking studies showed that her serum carried antibodies which prevented insulin from reacting with sensitized skin. Whether one and the same mechanism accounted for both the hormonal refractoriness and the antiallergenic power of her serum can be only surmised. Although such antibodies might well have engendered the original refractoriness, it is also theoretically possible that some unrelated mechanism was initially involved and that antibody production was only secondary to the unusual amounts of insulin given in an effort to overcome the refractoriness. In favor of the former hypothesis is the fact that the patient's blocking power per ml of serum was more than adequate to account for the large doses she was required to take daily. Whether, in this case, the interference with sugar metabolism was due to a direct union of antibody with the hormonally active groupings of the insulin molecule or was dependent upon complexing with other, nearby groupings which led to steric hindrance was not subject to experimental test. In the rabbit, the latter situation appears to be productive of insulin resistance according to Lowell (11).

The tendency for B. E. R.-resistant serum to delay rather than prevent hypoglycemic death

in the mouse suggests steric hindrance, although the formation of unstable antigen:antibody complexes might be an alternate explanation. The intermittent hypoglycemic shock experienced by the patient, at times many hours after her latest dose of insulin, might rest on instability of the hypothetical antigen:antibody complex, or on the release of insulin from some postulated reservoir.

The observation that blocking end-points with B. E. R. fractions were the same for unheated as for heated samples rules out the hypothesis that the blocking phenomenon is dependent upon heat-denatured reagin rather than on a separate, blocking antibody.

The use of serum:antigen mixtures for direct blocking tests in sensitized skin provided, in our experience, a more sensitive and practical means of measuring anti-insulin factor than the reagin-neutralization test with heated serum or the Lowell mouse-protection test—assuming that the same factor is being measured by all three procedures.

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