

IMMUNOPHYSIOLOGY OF SERUM SICKNESS

ALTERATIONS IN THE BLOOD VOLUME AND THIOCYANATE SPACE IN RELATION TO HUMORAL ANTIBODIES IN RABBITS SENSITIZED WITH HUMAN ALBUMIN OR GLOBULIN

JERRY K. AIKAWA, M.D.,* ERNEST H. YOUNT, M.D.,** (GEORGE T. HARRELL, M.D.),
AND TREVA M. JENNINGS, B.S.

THE physiologic disturbances resulting from immune reactions which follow the introduction of closely related purified antigens have received scant attention. Many years ago disturbances in the distribution of body fluids were observed clinically and alterations in renal function were detected during the course of serum disease.¹⁻⁴ With the development of chemical methods for separation of serum into fairly homogeneous fractions of reasonable purity, it has been possible to approach the problem of experimental hypersensitivity to foreign proteins in animals more definitively.

The histological and serological sequences in rabbits sensitized with albumin and globulin fractions of bovine serum have been described.⁵ The localization of protein antigens of various molecular sizes in the tissues of a heterologous animal by homologous antibody labeled with fluorescein has been reported.⁶

In a previous study it was found that rabbits sensitized by the intravenous injection of human plasma showed a decrease in the blood volume and an increase in the thiocyanate space which could be related to the time at which humoral antibody was first detected.⁷ In preliminary studies with whole plasma, serial determinations of the fluid volumes detected two separate alterations in the space available for dilution of thiocyanate ion. These observations suggested that the two peaks were the result of physiologic defects attributable to reactions to two separate antigens from the plasma. These results were analogous to the appearance of anatomic lesions at different times following the injection of two separate serum fractions.⁵

Accordingly, the present experiments were conducted to study the changes in distribution of fluids induced by antigen-antibody reactions resulting from purified fractions of human serum of different molecular size.

MATERIALS AND METHODS

Twenty-nine domestic rabbits of mixed breeds, weighing roughly two kilograms each, were used. Animals were placed in individual cages and fed

From the Department of Internal Medicine, The Bowman Gray School of Medicine, of Wake Forest College, Winston-Salem, North Carolina.

This work was supported (in part) by research grants from the John and Mary R. Markle Foundation, The National Heart Institute of the National Institutes of Health, Public Health Service, and the Atomic Energy Commission under a contract with the Bowman Gray School of Medicine.

*A.E.C. Fellow in the Medical Sciences of the National Research Council, 1948-1950.

**Markle Scholar in Medical Science.

a stock diet* supplemented once weekly by fresh green vegetables. Water was given without restriction.

Crystalline Human Serum Albumin.†—The very limited quantity of crystalline human serum albumin available was dissolved in sterile physiologic saline solution (2 Gm. in 10 ml.). No preservative was added and the solution was injected immediately without other sterilization. This solution was further diluted to a final concentration of 1:1000 in physiologic saline for use as antigen in the precipitin tests.

Human Serum Albumin.†—Concentrated salt-poor human normal serum albumin (25 Gm. in 100 ml.) was injected undiluted. The material was diluted 1:100 in physiologic saline for use as antigen in the precipitin tests.

Human Serum Globulin.†—Human immune serum gamma globulin (12.3 Gm. in 100 ml.) was injected undiluted. The material was diluted 1:100 in physiologic saline for use as antigen in the precipitin tests.

Only one lot of each of the antigenic materials was used to complete all of the experiments.

Chemical Techniques.—The plasma volume was determined by the T-1824 (Evans Blue) dye method. Syringes holding 2 milliliters were calibrated to deliver 0.5 ml. of a solution containing 3 mg. of the dye per milliliter. Two different lots of dye were used for the albumin and globulin experiments. The thiocyanate space was determined simultaneously. Syringes holding 10 ml. were calibrated and 2.0 ml. of a solution containing 50 mg. of sodium thiocyanate per milliliter were injected.

The concentrations of the dye in rabbit serum were determined by the Evelyn photoelectric microcolorimeter and the thiocyanate concentrations with the Evelyn photoelectric macrocolorimeter.⁷

Hematocrit readings were made on oxalated blood placed in Wintrobe tubes, centrifuged at 3,000 revolutions per minute at a radius of 15 cm. for 30 minutes.

The total blood volume was calculated from the plasma volume and the hematocrit.

Immunologic Techniques.—Undiluted rabbit serum was placed in 2 by 20 mm. tubes to a height of 1 cm. and overlaid with 0.5 milliliter of the diluted antigen. The tubes were left at room temperature and the presence or absence of precipitation at the interface was detected by observation in reflected artificial light against a dark background after an interval of one hour. Each tube was compared with a control.

Skin tests were not done in order to avoid additional injections of antigen. The dye T-1824 has previously been shown not to induce precipitins or positive skin tests in rabbits.⁷

Experimental Procedure.—In the morning before feeding, an ear vein was nicked with a knife and 2 ml. of blood collected in a dry test tube and allowed to clot; 1 ml. was then collected in another dry tube containing 6 mg. of ammonium oxalate and 4 mg. of potassium oxalate. The dye was injected into a

*Kasco Complete Rabbit Ration, Kasco Mills, Incorporated, Toledo, Ohio.

†Furnished by the American Red Cross.

TABLE I. MEAN OF TWO BASELINE AND TWO RESPONSE VALUES IN RABBITS SENSITIZED BY THE INTRAVENOUS INJECTION OF 0.5 GM. PER KG. OF CONCENTRATED HUMAN ALBUMIN (GROUP 2)

RABBIT NUMBER	BASELINE				HEMATOCRIT VOLS. %	RESPONSE*					HEMATOCRIT VOLS. %
	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML./KG.	PLASMA VOLUME ML./KG.	TOTAL BLOOD VOLUME ML./KG.		PRECIPITIN POSITIVE DAY	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML./KG.	PLASMA VOLUME ML./KG.	TOTAL BLOOD VOLUME ML./KG.	
1	2.50	157.6	44.4	76.4	42.0	8	2.50	172.0	40.0	63.6	37.5
2	2.27	179.3	39.2	63.0	37.5	7	2.61	226.8	29.9	46.0	36.0
3	2.50	163.0	34.8	58.0	40.0	7	2.64	206.8	29.9	48.5	38.5
4	2.52	146.8	40.8	69.0	41.0	8	2.75	143.6	33.8	55.3	38.5
5	2.27	170.5	45.8	72.2	37.0	8	2.52	165.1	35.7	54.4	34.5
6	2.33	165.2	36.1	60.9	41.0	9	2.60	185.0	28.5	46.5	39.0
7	2.50	155.2	47.2	77.2	40.0	5	2.66	178.9	37.9	61.3	39.0
8	2.64	158.7	42.4	75.0	44.0	6	2.81	177.2	31.3	50.5	40.0
9	2.35	160.4	40.0	65.5	39.5	7	2.66	186.5	30.8	48.9	36.5
10	2.16	184.3	37.5	63.9	41.5	6	2.50	203.6	28.8	45.2	36.5
11	2.70	146.3	35.6	58.9	39.5	7	2.84	168.0	29.9	46.1	35.5
12	1.93	189.6	42.5	66.8	36.5	7	2.39	200.8	32.2	49.0	34.0
Mean	2.39	164.7	40.5	67.2	40.0	7.1	2.62	184.6	32.4	51.3	37.1

*The mean of two consecutive determinations at the start of the experiment is compared with the mean of two consecutive determinations in the same rabbit done on the day precipitins to concentrated human albumin appeared and on the next day.

vein in the opposite ear, the syringe changed, and the sodium thiocyanate injected through the same needle within one minute. Ten minutes following completion of the injection, 2 ml. of blood were collected from the first ear. When serial determinations were previously done, this arbitrary mixing time was found to give reproducible results for both blood volume and thiocyanate space.⁸

Preliminary Experiments.—In order to establish the minimal sensitizing dose, 3 rabbits were given a single subcutaneous injection in the interscapular region of 0.5 Gm. per kilogram of concentrated human albumin. Two rabbits developed positive precipitin tests on the twenty-ninth day, but the third animal was still negative on the thirty-second.

A single subcutaneous injection of 0.75 Gm. per kilogram of concentrated human albumin was then given four other rabbits. Two animals developed positive precipitin tests at 13 days and 2 at 14 days following injection.

Definitive Experiments.—Two baseline determinations were made on successive days on each of 5 rabbits (Group 1). After the second determination, each animal was injected subcutaneously with a single dose of 0.75 Gm. per kilogram of crystalline albumin. Precipitin tests were performed daily on each rabbit, but no further fluid volume determinations were done until the day that a positive precipitin reaction was first obtained. The measurements were repeated on that day and the following day.

Twelve rabbits (Group 2) were treated in the same fashion except that a single intravenous injection of concentrated albumin in a dose of 0.5 Gm. per kilogram was given.

Six rabbits (Group 3) were studied in an identical manner except that 0.25 Gm. per kilogram of serum globulin was given intravenously.

Statistical Analysis.—The total blood volume, plasma volume, hematocrit, thiocyanate space and weight were recorded in absolute values. The mean of the two baseline determinations on each animal was compared with the mean of the two response determinations made on the day that precipitins appeared and on the following day. The mean differences between the baseline and response values were calculated. The calculated value for "t" was compared with the value for n-1 degrees of freedom as obtained from standard tables. The value was considered significant if P was less than 0.01.

The data obtained in absolute values were then calculated in terms of milliliters per kilogram of body weight and the statistical significance was determined. Each animal served as its own control. No attempt was made to compare one experimental group with another.

RESULTS

General Observations.—Clinical serum sickness, manifested by erythema or edema, was not observed in any of the groups with the doses used.

Crystalline Human Serum Albumin (Group 1).—Positive precipitin tests appeared in all animals at a mean of 22.5 days. No significant changes were found in any of the response values when they were compared with the baseline values.

Human Serum Albumin (Group 2).—Positive precipitin tests developed in all animals at a mean of 7.1 days. The alterations in thiocyanate space, plasma volume, total blood volume, hematocrit and body weight are summarized in Table I, the statistical analyses in Table II.

TABLE II. STATISTICAL SIGNIFICANCE OF ALTERATIONS IN FLUID SPACES IN RABBITS SENSITIZED WITH CONCENTRATED HUMAN ALBUMIN

	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML.	PLASMA VOLUME ML.	TOTAL BLOOD VOLUME ML.	HEMATOCRIT VOL. %
$\bar{d}$	+ .230	+91.92	-11.40	-26.40	-2.83
$s_{\bar{d}}$	.035	13.50	1.44	3.12	.37
t	6.570	6.81	7.92	8.46	7.65
		ML./KG.*	ML./KG.*	ML./KG.*	
$\bar{d}$		+19.82	-8.08	-16.04	
$s_{\bar{d}}$		4.50	.20	1.10	
t		4.40	40.40	14.58	

*Calculated in terms of weight on the day of determination.

$\bar{d}$ = mean difference

$s_{\bar{d}}$ = standard error of the mean difference

S = summation of, n = number of animals.

$$t = \frac{\bar{d}}{s_{\bar{d}}} \quad s_{\bar{d}} = \sqrt{\frac{Sd^2 - \frac{(Sd)^2}{n}}{n(n-1)}}$$

For 11 degrees of freedom $t = 3.106$; $P = <.01$

The data all show statistically significant differences between the baseline values and those obtained at the time of the humoral antibody response.

A significant increase in the mean thiocyanate space was noted at the time the humoral antibody was first detected. A significant increase in body weight occurred during the period of alteration in thiocyanate space. In contrast to the increase in thiocyanate space and weight, significant decreases in plasma volume, total blood volume, and hematocrit occurred in all animals.

All the data are statistically significant whether the calculations are based on the comparison of absolute values or of values based on milliliters per kilogram of body weight.

Human Serum Globulin (Group 3).—All animals developed positive precipitin tests at a mean of 9.0 days. The alterations in thiocyanate space, plasma volume, total blood volume, hematocrit and body weight are summarized in Table III, the statistical analyses in Table IV.

The increase in thiocyanate space was significant only when the data were analyzed in absolute values. The increase in body weight was significant. The decreases in plasma volume, total blood volume and hematocrit were significant whether the data were calculated in absolute values or in terms of milliliters per kilogram of weight.

DISCUSSION

The observations reported demonstrate that immune reactions to albumin and globulin fractions of human serum are associated with alterations in distribution of fluids within the body. The changes were detected earlier and were quantitatively greater after the injection of albumin than after globulin, but qualitatively they were the same. The absence of clinical manifestations of serum disease in our animals suggests that the disease was mild. The variation in the time of appearance of precipitins in relation to the experiments of others

TABLE III. MEAN OF TWO BASELINE AND TWO RESPONSE VALUES IN RABBITS SENSITIZED BY THE INTRAVENOUS INJECTION OF 0.25 GM. PER KG. OF HUMAN GAMMA GLOBULIN (GROUP 3)

RABBIT NUMBER	BASELINE					RESPONSE*					
	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML./KG.	PLASMA VOLUME ML./KG.	TOTAL BLOOD VOLUME ML./KG.	HEMATOCRIT VOLS. %	PRECIPITIN POSITIVE DAY	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML./KG.	PLASMA VOLUME ML./KG.	TOTAL BLOOD VOLUME ML./KG.	HEMATOCRIT VOLS. %
13	1.96	250.0	56.1	93.4	39.0	9	2.10	261.9	46.2	74.3	36.5
14	2.09	234.0	58.4	101.0	41.0	7	2.33	215.5	46.4	79.8	39.5
15	1.91	266.0	67.5	103.1	35.0	9	1.93	302.6	57.0	85.0	33.5
16	1.56	313.5	69.2	112.8	36.5	10	1.70	329.4	52.9	89.4	34.5
17	1.93	273.6	64.8	106.2	39.0	8	2.05	275.6	55.1	81.5	34.0
18	2.10	262.8	70.0	103.8	42.0	11	2.18	278.8	56.0	79.3	39.0
Mean	1.93	233.3	64.3	103.4	38.8	9.0	2.05	277.3	52.3	81.6	36.2

*The mean of two consecutive determinations at the start of the experiment is compared with the mean of two consecutive determinations in the same rabbit done on the day precipitins to human gamma globulin appeared and on the next day.

TABLE IV. STATISTICAL SIGNIFICANCE OF ALTERATIONS IN FLUID SPACES IN RABBITS SENSITIZED WITH HUMAN GAMMA GLOBULIN

	WEIGHT IN KILOGRAMS	THIOCYANATE SPACE ML.	PLASMA VOLUME ML.	TOTAL BLOOD VOLUME ML.	HEMATOCRIT VOLS. %
$\bar{d}$	+0.122	+52.20	-16.80	-32.00	-2.58
$s_{\bar{d}}$	0.028	9.60	1.99	3.40	0.54
t	4.370	5.43	8.44	9.40	4.78
		ML./KG.*	ML./KG.*	ML./KG.*	
$\bar{d}$		+10.70	-12.23	-21.80	
$s_{\bar{d}}$		7.40	1.37	1.14	
t		1.45	8.93	19.10	

*Calculated in terms of weight on the day of determination.

For 5 degrees of freedom, $t = 4.032$; $P = <.01$.

With the exception of the thiocyanate space in ml. per kg. the data all show statistically significant differences between the baseline values and those obtained at the time of the humoral antibody response.

may be due to differences in the species of serum utilized as antigen. Other investigators have used chiefly horse or bovine serum.^{4, 5, 9} The preliminary experiments indicate that the size of the dose affects the incubation period, but not the severity of the disease.

The crystalline serum albumin used may well have been antigenically altered in the process of crystallization. The appreciably longer period before precipitin tests became positive, as compared with concentrated serum albumin, is suggestive of impaired antigenicity. Another factor contributing to the longer incubation period may have been the difference in the route of administration of the antigen. The fact that significant physiologic alterations were not detected may be due to either of these factors, or to both.

The increase in space available for dilution of the thiocyanate ion at the time circulating humoral antibodies appeared, when compared with the control period in the same animal, indicates that this small molecule diffused into some extravascular space to a greater extent. The loss of water and ions from the vascular tree into tissues would readily explain the observations of retention by the body of water, salt and chlorides during serum disease and pneumonia.^{2, 10} The decrease in circulating blood volume confirms the suggestion that particles had left the circulation.⁷ The colloids lost to the interstitial spaces would tend to retain water extravascularly until they had been reabsorbed or broken down in the tissues. It is well known that in severe immune reactions of the anaphylactoid type hemorrhages, due to the extravasation of the even larger red blood cell, appear in various organs.

The observations can be interpreted as indicating an increase in permeability of the vascular tree, so that crystalloids, colloids, and red blood cells are progressively lost from the circulation as the magnitude of the defect increases with increase in the clinical severity of the disease. The alterations in the vascular tree can be visualized as a series of pores, probably in the capillary wall where the normal extravascular circulation of crystalloids, and to a lesser extent small protein molecules such as albumin, is known to occur. In mild instances of serum disease water and ions which normally traverse the membranes would

leave the blood more readily. Larger molecules such as protein would be lost in more severe instances and red blood cells would pass out of the vascular tree only in the most severe reactions.

A uniform, significant reduction in the hematocrit occurred in all animals in Groups 2 and 3. Since the plasma volume had decreased, the total circulating red cell mass must have decreased also. There was no clinical evidence of increased capillary permeability to red cells as evidenced by hemorrhage or petechiae. The clinical disease induced appeared to be too mild to account for the decrease in hematocrit on the basis of extravasation of red cells out of the vascular tree. The possibility of hemolysis must be considered; jaundice has been previously observed in another group of animals sensitized with human plasma. No such sign was detected in the present study. It is also possible that a shunting and pooling of red cells in some inactive tissue or organs had occurred. No adequate explanation can be given at the present time for this reduction in hematocrit.

The proportionately smaller increase in thiocyanate space in the animals injected with globulin, as compared with those injected with albumin, may have been due to the fact that the antigenic stimulus was quantitatively less. A smaller dose was administered and the larger size of the molecule would mean that fewer antigenic particles were injected in a given weight of serum. Furthermore, the globulin molecules may be less antigenic than the smaller albumin molecules. The reduction in the incidence of clinical serum disease noted since the introduction of therapeutic antisera composed chiefly of the gamma globulin fraction lends further support to this hypothesis. The cells to which antigens of various size attach, as well as the speed of attachment, vary with the type of antigen injected.⁶ The globulin molecule may attach to cells which have less capacity for the production of antibodies.

The increase in extravascular fluid space as measured by the thiocyanate ion could not always be correlated with an increase in weight. The alterations cannot therefore be attributed solely to a defect in capillary permeability with retention of extra fluid in the interstitial spaces. Apparently the normal distribution of fluid in the body was altered, probably as the result of an increase in permeability of cellular membranes as well. It is not known whether the thiocyanate ion normally diffuses into cells or not. In any event these observations indicate that at the time humoral antibodies were detected the permeability of membranes to these ions was increased, so that they diffused from blood into interstitial spaces and probably from interstitial fluid spaces into cells.

Histological techniques have demonstrated that after the injection of antigens cellular reactions can be detected by anatomic methods before humoral antibodies are present.^{5, 9} It has been presumed that these histological changes are the result of antigen-antibody reactions taking place on the cell membrane or within the cell, but the speed with which specific antibodies are formed within cells or attached to them is not known.

The role of antigen-antibody reactions in the production of anatomic glomerular lesions following the injection of bovine globulin into rabbits has

recently been questioned.⁹ The fact that anatomic lesions appear before humoral immunity is detectable suggests the alternative possibility that physiochemical relationships concerned with the hydration of intraglomerular proteins may effect changes without an immune reaction taking place.

Correlation of these physiologic experiments with previous histologic studies could not always be made. The injection of albumin fractions of bovine or horse serum results in generalized arterial lesions; the globulin fractions produce glomerular lesions more frequently.^{5, 9} It is likely that the physiologic defect which we have studied occurs not in the artery, but in the capillary for reasons given earlier. The physiologic defect reaches its maximum after the anatomic lesion appears, is completely reversible if the animal lives, and disappears at a time when the anatomic lesion may persist.^{5, 7} In rickettsial diseases reversible physiologic changes may be present which are out of all proportion in magnitude to the number of anatomic vascular lesions present.¹¹ The similarity of the histological picture in rickettsial diseases and serum sickness suggest that some common denominator may be present. An immune reaction is such a factor.

SUMMARY

1. Alterations in the distribution of fluids associated with the development of humoral immunity were detected in rabbits injected intravenously with human serum albumin or globulin.

2. Increase in the space available for dilution of thiocyanate ions and decreases in plasma volume, total blood volume and hematocrit occurred at the time circulating antibody (precipitins) appeared.

3. The changes appeared earlier (at a mean of 7.1 days) following the injection of albumin than after the injection of globulin (9.0 days). Crystalline human serum albumin, injected subcutaneously, did not produce significant physiologic disturbances and the appearance of precipitins was delayed for a mean of 22.5 days. These findings were interpreted as indicating an alteration in antigenicity of the protein in the process of crystallization, but may have been related to the subcutaneous route of injection.

4. The redistribution of fluid was interpreted as indicating a defect in permeability of capillaries and of cell walls as a result of antigen-antibody reactions.

5. The correlation with anatomic findings by histologic techniques is discussed.

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