THE EFFECT OF RHEUMATOID FACTORS AND OF ANTIGLOBULINS ON IMMUNE HEMOLYSIS IN VIVO*

By MANUEL E. KAPLAN,‡ M.D., AND JAMES H. JANDL, M.D.

(From the Thorndike Memorial Laboratory and Second and Fourth (Harvard) Medical Services, Boston City Hospital, and the Department of Medicine, Harvard Medical School, Boston)

(Received for publication, August 29, 1962)

The sera of patients with rheumatoid arthritis frequently contain elevated concentrations of gamma globulins that have sedimentation coefficients, on ultracentrifugation, of about 19 Svedberg units (19S) (1, 2). These macroglobulins, collectively known as "rheumatoid factors," are recognized by their ability to react with 7S gamma globulins under appropriate conditions *in vitro* (3–7). Because these characteristics suggest immunologic capabilities it has been postulated that these substances are antibodies to gamma globulin and that their presence signifies an "auto-immune" state (7–10). To date no direct evidence has been obtained to implicate the rheumatoid factors in the genesis of rheumatoid arthritis (10, 11). Nevertheless, speculation continues regarding their origin and possible functions.

The studies herein described were undertaken in an attempt to determine whether rheumatoid macroglobulins act like known heterologous antibodies to human gamma globulin and whether they function *in vivo*. To this end, their effects *in vitro* and *in vivo* on red cells sensitized with incomplete antibodies were observed and compared with those produced by immune antiglobulins.[1]

Materials and Methods

I. Studies in Rats:

A. Immunization of Rats.—Fresh blood was obtained from the lateral ear veins of 2 white male albino rabbits and from the hearts of male Sprague-Dawley rats of a caesarian-derived, *Bartonella*-free strain.[2] The blood specimens were allowed to clot for 1 hour at 37°C, and the sera separated by centrifugation and stored in 2 ml aliquots at −20°C until used.

* This investigation was supported in part by Grant GM-03507-11 from the National Institutes of Health, Bethesda.

‡ Postdoctoral Fellow, the National Heart Institute, United States Public Health Service. Present address: Department of Hematology, Mount Sinai Hospital, New York.

[1] A preliminary report of this work has previously been published (12).

[2] Charles River Laboratories, Boston.

The recipient animals were large (400 to 550 gm) males of the same strain as the donor rats. They were paired by weight and one of each pair received, by injection into the foot-pads and into multiple subcutaneous sites, 1.0 ml of rabbit serum thoroughly emulsified in 1 ml of complete Freund's adjuvant.[3] The paired control animals received, in an identical fashion, 1.0 ml of isologous rat serum in adjuvant. Weekly thereafter, for 6 weeks, 1.0 ml of the appropriate serum without adjuvant was administered intraperitoneally. The red cell survival studies, to be described below, were performed within 2 to 4 weeks after the last serum injection.

B. Sensitization of Rat Red Cells.—Rat red cells from isologous donors were labeled with Cr^{51}[4] and sensitized with non-agglutinating quantities of rabbit antirat red cell antiserum as previously described (13). The agglutinating and sensitizing characteristics of this antiserum are depicted in Table I.

C. Red Cell Survival Studies.—In each experiment 4 animals were studied simultaneously, 2 rats immunized with rabbit serum and 2 that had received rat serum. Before the survival

TABLE I

The effect of Rabbit Anti-Rat Red Cell Serum (7–59) on Rat Red Cells in Vitro

Rabbit antiserum, *ml* / Rat red cells, *ml*	Hemolysis in fresh rat serum	Agglutination	
		In serum	In PVP.*
0.000	0	0	0
0.020	0	0	2 to 3+
0.027	0	0	3+
0.050	0	0	3 to 4+
0.080	0	1+	4+
0.133	0	2 to 3+	4+
0.200	0	3 to 4+	4+

* PVP, a buffered (pH 7.4) solution of 5 per cent polyvinylpyrrolidone, K-44.

studies were begun, 0.2 ml of whole blood was taken by tail puncture from each animal into capillary tubes. The blood was allowed to clot and the serum separated by centrifugation. The undiluted sera were tested for their ability to agglutinate a 5 per cent suspension of the sensitized red cells whose survival was to be studied. The sensitized cells were then injected, their survival and sites of sequestration determined, and the results calculated and expressed as described in a previous publication (14). At the time of sacrifice, 3 hours after injection, the animals were exsanguinated by cardiac puncture and the derived sera were collected.

In two experiments a 50 per cent suspension of Cr^{51}-labeled rat cells sensitized with rabbit antiserum was secondarily incubated for 1 hour, with an equal volume of pooled, undiluted serum from rats that had been immunized with rabbit serum or that had been comparably injected with rat serum. After incubation, the degree of agglutination was determined and the survival and sequestration of these doubly incubated cells were measured in normal, non-immunized rats.

---

[3] Difco Laboratories, Detroit.

[4] $Na_2Cr^{51}O_4$, 300–500 mc/mg; Abbott Laboratories, North Chicago.

II. *Studies in Man:*

A. Materials.—

1. Red cells. Whole blood was obtained as required from a single normal donor of blood group O, R_1R_1 (CCDee) and mixed with 1/4 volume of sterile ACD.[5] The red cells were used within a few hours of the time the blood was drawn.

2. Incomplete anti-Rh antisera. Plasma from 3 hyperimmunized donors was used. Their individual serological characteristics and reactivities with normal donor red cells are illustrated in Table II.

3. Rheumatoid sera. Sera were obtained from 35 patients with rheumatoid arthritis exhibiting strongly positive latex fixation tests[6] and were stored at −20°C until used.

4. Rabbit antiserum. Rabbit antiserum against whole human serum was prepared by repeatedly injecting a single albino rabbit with normal human serum according to the method of Emerson *et al.* (15). Prior to use the antiserum was heated for 30 minutes at 56°C and ab-

TABLE II

The Serological Characteristics in Vitro of Various Anti-Rh Antisera versus Type 0, R_1R_1 Red Cells

Source of antiserum	Specificity	Saline agglutinin titer	Indirect anti-globulin titer	Gm (a)* type
Ham	Anti-D	0	1:256	+
Ges	Anti-CD	Tr	1:512	−
Mur‡	Anti-CDE	1:2	1:512	−§

* We are grateful to Dr. Hugh Fudenberg for Gm-typing many of the sera employed.

‡ Anti-Rh Murphy supplied by Knickerbocker Laboratories, New York.

§ The Gm type of Mur serum was reported by Harboe (34) to be Gm (a − b+ x−).

sorbed twice with 2 volumes of washed, packed human red cells pooled from type A_1, B, O, and AB donors. The resulting serum, which did not agglutinate normal human red cells, was stored in 2.0 ml aliquots at −20°C. As required, aliquots were thawed and sterilized by passage through a Millipore filter[7] of pore size 0.45 μ.

5. Diluent. Unless stated otherwise, unbuffered 0.85 per cent saline was employed in all studies.

B. Methods.—

1. Sensitization of red cells with anti-Rh antisera. Red cells from the normal donor were washed twice in large volumes of saline and brought to a 50 per cent concentration in saline. To 1 volume of the 50 per cent red cell suspension was added either (*a*) 1 volume "Ham.", (*b*) 1/5 volume "Ges.", or (*c*) 1/5 volume "Mur." These amounts of the various sera were selected because they had been found in preliminary experiments to produce comparable rates and patterns of sequestration of Rh-positive red cells *in vivo*. Following incubation for 1 hour

[5] ACD, acid citrate dextrose mixture, Abbott Laboratories, North Chicago.

[6] The cooperation of Dr. Ellis Dressner, Dr. Sumner Frank, Dr. Alan Cohen, and Dr. Arthur Hall is gratefully acknowledged.

[7] Millipore Filter Corporation, Bedford, Massachusetts.

at 37°C, the cells were washed thrice and suspended in sufficient saline to provide a 5 per cent red cell concentration for *in vitro* studies or a 50 per cent red cell concentration for *in vivo* studies.

2. Agglutination studies. Two fold serial dilutions of the rheumatoid sera, ranging from neat serum to 1:16,384, were prepared in 0.2 ml quantities. To each dilution was added an equal volume of a given 5 per cent suspension of sensitized red cells. After incubation for 2 hours at room temperature the cells were resuspended by gently tapping the tubes and the intensity of red cell agglutination was observed macro- and microscopically. The degree of agglutination was categorized as follows: 1+ = microscopic agglutination of 5 to 10 per cent of cells; 2+ = microscopic agglutination of 20 to 35 per cent of cells; 3+ = macroscopic agglutination involving more than 50 per cent, but less than 75 per cent, of the cells; 4+ = gross macroscopic clumping involving more than 75 per cent of all red cells. Five of the 35 rheumatoid sera were selected for further study because they induced unusually strong agglutination (more than "3+") of red cells coated with one or more of the antisera (Table III).

3. Absorption studies. To a volume of rheumatoid serum "Cor.," (*a*) undiluted and (*b*) diluted 1:50 in saline, were added 3 volumes of packed red cells sensitized with anti-Rh antiserum Ges. As controls, unsensitized red cells were added in an identical fashion to neat and dilute rheumatoid serum Cor. Following incubation for 1 hour at room temperature, the cells were packed by centrifugation and the supernatant sera were removed. The latter were reincubated with packed cells as before and, thereafter, the entire procedure repeated for a third time. The supernatants from the third incubation were titered for rheumatoid factor activity against a 5 per cent suspension of Ges.-sensitized red cells in the usual fashion.

4. Agglutination-inhibition studies. To each of four series of test tubes containing 0.05 ml of packed red cells previously sensitized with Ges. was added 0.95 ml of either serially diluted human serum albumin[8] (the initial tube containing an albumin concentration of 5 gm per cent) or serially diluted serum from (*a*) a normal individual, (*b*) a hyperglobulinemic patient with multiple myeloma, and (*c*) a patient with acquired agammaglobulinemia. The cells were dispersed thoroughly in the suspending media, and 1.0 ml of a 1:64 dilution of rheumatoid serum Cor. was pipetted into each tube. After incubation for 2 hours at room temperature, each tube of the four series of tubes was examined for red cell agglutination.

5. Survival studies of sensitized red cells. The destruction *in vivo* of Rh-sensitized red cells was studied in (*a*) a group of 7 control subjects (for the most part elderly, male alcoholics without clinical evidence of liver disease), and (*b*) in 5 patients with rheumatoid arthritis[9] whose sera strongly agglutinated the sensitized red cell preparations employed. 25 ml of normal blood was collected in sterile non-pyrogenic ACD and labeled with 150 to 250 μc. of Cr^{51} as previously described (16). Thereafter, the cells were washed, sensitized with one of the three anti-Rh antisera employed, and rewashed thoroughly (as described in section II-B (1)). The absence of red cell agglutination was confirmed microscopically before infusion of the cells into the recipient. The survival of these sensitized red cells was measured as in previous studies (17) utilizing external body scanning over the precordium, liver, and spleen to localize red cell sequestration.

When so indicated, the Cr^{51}-labeled sensitized red cells were subjected to a second incubation *in vitro*, either with sterile rheumatoid serum previously obtained from the subject under study or with sterile rabbit antiserum. In the first instance, a 50 per cent suspension in saline of sensitized Cr^{51}-labeled red cells that had been washed thrice in saline was added to equal

[8] Nutritional Biochemicals Corporation, Cleveland.

[9] We are indebted to Dr. Mortimer S. Greenberg and Dr. Marvin L. Mitchell of the Lemuel Shattuck Hospital, Boston, for making several of these studies possible.

TABLE III

Agglutination of Rh-Sensitized Red Cells by Five Rheumatoid Sera

Rheumatoid serum dilution 1:	Source of rheumatoid serum												
	Cor.			Mar.			Ing.			West.			Dick.
	Anti-Rh coat			Anti-Rh coat			Anti-Rh coat			Anti-Rh coat			Anti-Rh coat
	Ham.	Ges.	Mur.	Ham.	Ges.	Mur.	Ham.	Ges.	Mur.	Ham.	Ges.	Mur.	Ges.
0	1+	0	±	0	0	±	±	0	±	±	0	±	1+
2	2+	0	1+	0	±	±	±	0	0	±	±	0	0
4	2 to 3+	0	2+	±	2+	1+	±	±	0	±	±	0	0
8	3 to 4+	1 to 2+	3+	±	3+	2+	1+	1+	0	1+	2+	0	±
16	4+	2 to 3+	3 to 4+	1+	3 to 4+	2 to 3+	±	2+	±	2+	3+	±	1+
32	4+	3 to 4+	4+	1+	4+	3+	±	2 to 3+	1 to 2+	2+	4+	2+	1 to 2+
64	4+	4+	4+	1 to 2+	3 to 4+	3 to 4+	0	3+	2+	2+	4+	2+	2+
128	4+	4+	4+	2+	3+	2+	0	3 to 4+	2 to 3+	1 to 2+	4+	2+	2 to 3+
256	4+	4+	4+	1+	2+	1 to 2+	0	3 to 4+	2+	1+	3 to 4+	2+	2+
512	4+	4+	4+	±	1 to 2+	1+	0	3+	1 to 2+	1+	2 to 3+	1 to 2+	1 to 2+
1024	4+	4+	4+	0	±	±	0	1 to 2+	±	±	1 to 2+	±	1+
2048	4+	3 to 4+	3 to 4+	0	0	0	0	±	0	±	1+	0	±
4096	3+	2+	2+	0	0	0	0	0	0	0	0	0	0
8192	1+	±	±	0	0	0	0	0	0	0	0	0	0
16,384	0	0	0	0	0	0	0	0	0	0	0	0	0

volumes of rheumatoid serum (undiluted or diluted, as specified). After incubation for 1 hour at room temperature, the cells were thoroughly dispersed by repeated inversion of the container, examined for agglutination, and injected promptly into the recipient without prior washing. Incubation of sensitized red cells with the heterologous rabbit antiserum was performed in an identical fashion with the exception that 10 volumes of a 50 per cent suspension of unlabeled type O, Rh-negative (rr) red cells were added to the sensitized Cr^{51}-labeled cells prior to the addition of the rabbit antiserum; this step was taken to retard formation of large agglutinates that might cause untoward reactions. No such reactions were observed in any of the subjects studied.

RESULTS

I. Studies in Rats:

When rat red cells lightly sensitized with rabbit antibody were suspended in normal rat serum or in the sera of rats that had been injected repeatedly with rat serum, no agglutination or hemolysis took place. After their injection into such "control" rats, these lightly sensitized cells were cleared relatively slowly from the circulation, and were sequestered principally in the spleen (Fig. 1). In one instance the amount of antiserum employed was so small that the subsequent survival of the sensitized cells was essentially normal (Fig. 1 B). When rat red cells, similarly sensitized with rabbit antibody, were suspended in the sera of rats that had been immunized against rabbit serum, there was no hemolysis, but agglutination did occur, as expected. Following their injection into such rats these sensitized cells were cleared from the circulation much more rapidly and completely, and the main site of sequestration was the liver (Fig. 1). It is apparent that the degree and velocity of sensitized red cell destruction observed in an individual animal immunized with rabbit serum correlated well with the capacity of the animal's serum to agglutinate the injected cells.

That the enhanced destruction of sensitized red cells in rats immunized with rabbit serum is serologically determined was clearly demonstrated in a subsequent study. Prior to their injection, the sensitized rat red cells were incubated with pooled serum from rats immunized with rabbit serum. When this preparation was infused into normal animals, the kinetics of red cell sequestration mimicked that obsesrved following injection of sensitized red cells into actively immunized animals (Fig. 2).

II. Studies in Man:

A. Rheumatoid Factors.—

In vitro observations. As indicated in Table III the agglutination by a given rheumatoid serum of type O, R_1R_1 red cells sensitized with any of three Rh-antisera is characterized by (*a*) great variability in the resultant intensity of agglutination depending upon the source of coating antibody and (*b*) prozones that are invariably present and extraordinarily broad. The specificity of the reaction between rheumatoid factors and the anti-Rh coating antibodies has been attributed to the existence of genetically determined differences among

human 7S gamma globulins (18). The latter, on the strength of this finding, have been subdivided into the so called "Gm-types." In the present studies the Gm specificities of the reactive components were identified only in certain

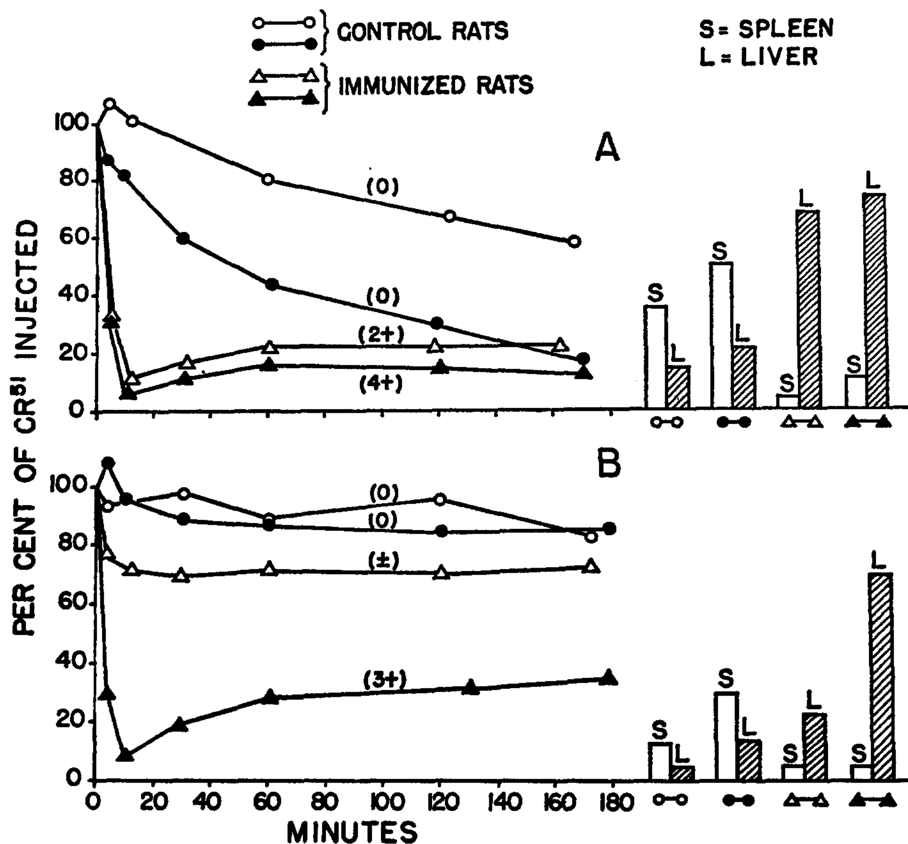

FIG. 1. The survival and sites of sequestration in rats of Cr^{51}-labeled rat red cells sensitized with rabbit antiserum. In Fig. 1 A the ratio of antiserum/packed cells was 0.050; in Fig. 1 B it was 0.027. The "immunized" rats (triangles) had been injected previously with rabbit serum and their sera contained anti-rabbit globulins in variable amounts. The "control" rats (circles) had been injected in a comparable manner with homologous rat serum. The degree of agglutination of sensitized cells in each recipient's serum is represented by a figure in parentheses (0 to 4+) above the respective survival curve.

instances. It was sufficient for our purposes to determine to what degree a given rheumatoid serum would agglutinate red cells coated with a specific antiserum. However, the nature of the prozone phenomenon was investigated more fully. It was found that a prozone was invariably present whenever a rheumatoid

serum interacted with sensitized red cells to produce agglutination.[10] Despite the testing of rheumatoid sera from 35 separate patients we were unable to find a single serum that, in the undiluted state strongly agglutinated red cells sensitized with any of the 4 Rh-antisera studied. The prozones that developed

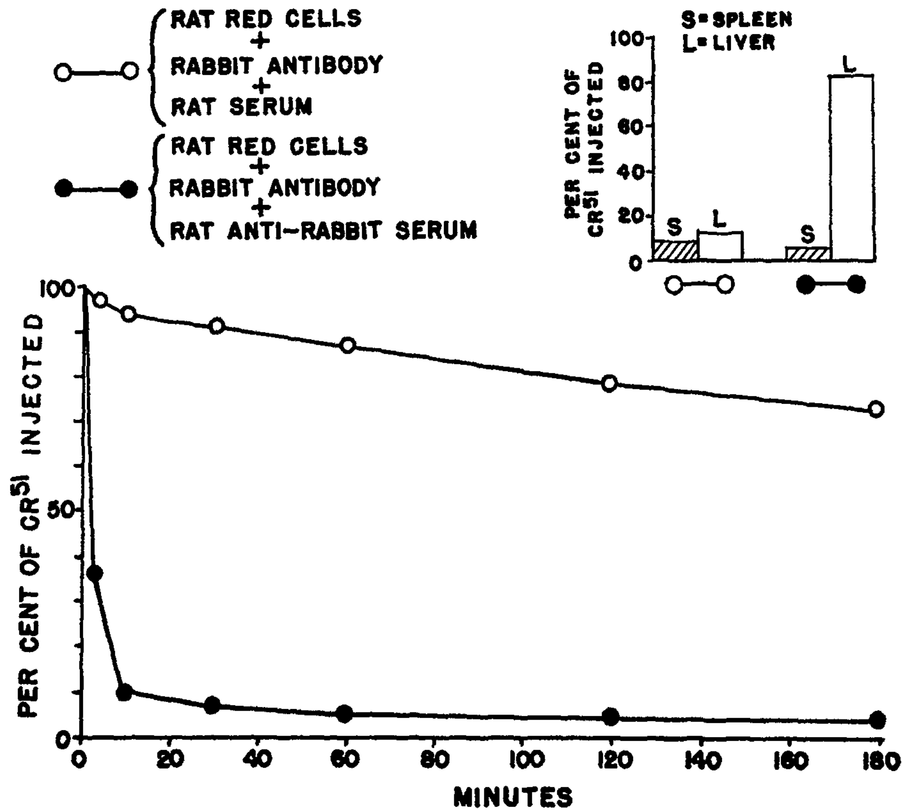

FIG. 2. The survival and sites of sequestration in normal rats of Cr^{51}-labeled, antibody-treated red cells preincubated *in vitro* with "control" rat serum or with rat serum containing antibodies against rabbit serum. Each point represents the mean of 2 observations.

when Rh-sensitized red cells were incubated in rheumatoid sera differed significantly from those frequently observed with incubation of similarly sensitized red cells in heterologous antiglobulin (Coombs) serum. Thus, with rheumatoid sera prozones were invariable and marked and no correlation could be observed between the prozone range and the agglutination titer, whereas in the Coombs

[10] In addition to the three anti-Rh sera described in detail, a small supply of anti-Rh "Ripley" was generously supplied to us by Dr. Ellis Dressner, and was utilized in similar serological titration studies.

reaction, prozones occur irregularly, often weakly and usually appear when the antiglobulin concentration is very high and the quantity of cell-fixed globulin is small; *i.e.*, there is a marked antiglobulin excess (19). In several of the rheumatoid sera studied, prozones were so marked that there were more tubes in the lower dilution range that lacked any agglutination than there were tubes showing agglutination. Furthermore, it was observed that the mere addition of saline to a suspension of sensitized red cells in the prozone range produced prompt agglutination. Once agglutinated, the cells could be washed in saline repeatedly without disagglutinating. The simplest explanation for the prozone phenomenon observed here is that serum contains a factor or factors that either (*a*) inhibit the interaction of rheumatoid factor with cell-fixed antibody or (*b*) stabilize

TABLE IV

The Agglutination of Rh-Sensitized Red Cells by Rheumatoid Serum—Inhibition by Various Sera Containing Different Concentrations of γ-Globulin

Source of serum	γ-globulin concentration	Dilution of inhibitory sera 1:											
		0	2	4	8	16	32	64	128	256	512	1024	2048
	gm per cent												
Normal	1.05	0	0	0	2+	3 to 4+	4+	4+	4+	4+	4+	4+	4+
Agammaglobulinemic	0.025	1+	3+	3 to 4+	4+	4+	4+	4+	4+	4+	4+	4+	4+
Hypergammaglobulinemic	9.38	4+*	1+*	0	0	0	0	±	2 to 3+	3 to 4+	4+	4+	4+

* Agglutination in these first two tubes is attributable to the non-specific effect of high concentrations of rouleaux-inducing globulins (36).

the product of this reaction. To investigate these possibilities the following experiments were performed:

1. Agglutination inhibition studies: The addition of human serum albumin, in various concentrations up to 5 gm per cent, to a system containing Ges.-sensitized red cells and dilute rheumatoid serum (Cor. 1:64) failed to inhibit red cell agglutination. When similar studies were performed, substituting for serum albumin human sera containing different quantities of γ-globulin, variable prozones resulted—the degree of inhibition produced by a given serum being directly proportional to its γ-globulin content (Table IV).

2. Absorption studies: Repeated incubations of rheumatoid sera with packed sensitized red cells demonstrated that agglutinating activity was not specifically absorbed from undiluted rheumatoid serum. On the other hand, significant absorption did occur in the dilute system, as evidenced by diminished intensity of red cell agglutination and a fall in absolute titer (Table V).

In vivo observations. The survival of Cr^{51}-labeled type O, R_1R_1 red cells sensitized *in vitro* with various Rh-antisera was measured in 7 control subjects and in the 5 patients with rheumatoid arthritis whose serum, when appropri-

ately diluted, most strongly agglutinated the particular sensitized red cell preparation to be infused. Two control subjects and 2 rheumatoid patients were studied on more than one occasion. Prior studies (17) have shown that in

TABLE V

Titration of Residual Rheumatoid Factor Activity after Absorption of Rheumatoid Serum with Packed Red Cells

Dilution of rheumatoid serum absorbed	Red cells	Dilution of absorbate 1:														
		0	2	4	8	16	32	64	128	256	512	1024	2048	4096	8192	16,384
0	Sensitized	0	±	1+	2+	3+	4+	4+	4+	4+	4+	3 to 4+	2+	±	0	0
	Control	0	0	1+	2+	3 to 4+	4+	4+	4+	4+	4+	2 to 3+	1+	0	0	0
1:50	Sensitized	3+	4+	4+	3 to 4+	2+	1+	0	0	0	0	0	0	0	0	0
	Control	4+	4+	4+	4+	4+	3 to 4+	2+	1+	±	0	0	0	0	0	0

TABLE VI

The Sequestration of Rh-Sensitized Red Cells in Human Subjects

Subject	Anti-Rh	Cr^{51} survival t ½, min.	CPM Spleen/liver
Normal			
Bay	Ges.	32.0	3.0
O'Mal	"	29.0	6.5
Corz	"	19.5	3.9
Murp	Ham.	26.5	1.2
Boy	"	17.0	5.4
Bronz	"	35.0	1.1
Bail	Mur.	22.0	3.6
Rheumatoid arthritis			
Mar.*	Ges.	4.2	3.9
Dick	"	17.0	4.1
Ing	"	19.5	1.8
West	"	27.5	3.1
Cor.(*a*)	"	22.5	2.3
Cor. (*b*)	Ham.	23.0	1.7
Cor. (*c*)	Mur.	29.5	1.3

* Patient Mar. has marked splenomegaly and clinical evidence of "hypersplenism."

normal individuals the usual half-survival time of such sensitized red cells ranges from 17 to 35 minutes with splenic far exceeding hepatic sequestration. The results obtained in the present control group are in accord with the previous findings (Table VI, Fig. 3). In 4 of the 5 patients with rheumatoid arth-

ritis studied, the rates of disappearance and patterns of sequestration of the sensitized cells fell entirely within the normal range (Table VI, Fig. 4). The fifth patient (Mar.) cleared his circulation of sensitized red cells with abnormal rapidity ($t\ 1/2$ = 4 to 5 minutes), with splenic sequestration predominating. However, this patient's clinical status differed from that of the other 4 in that

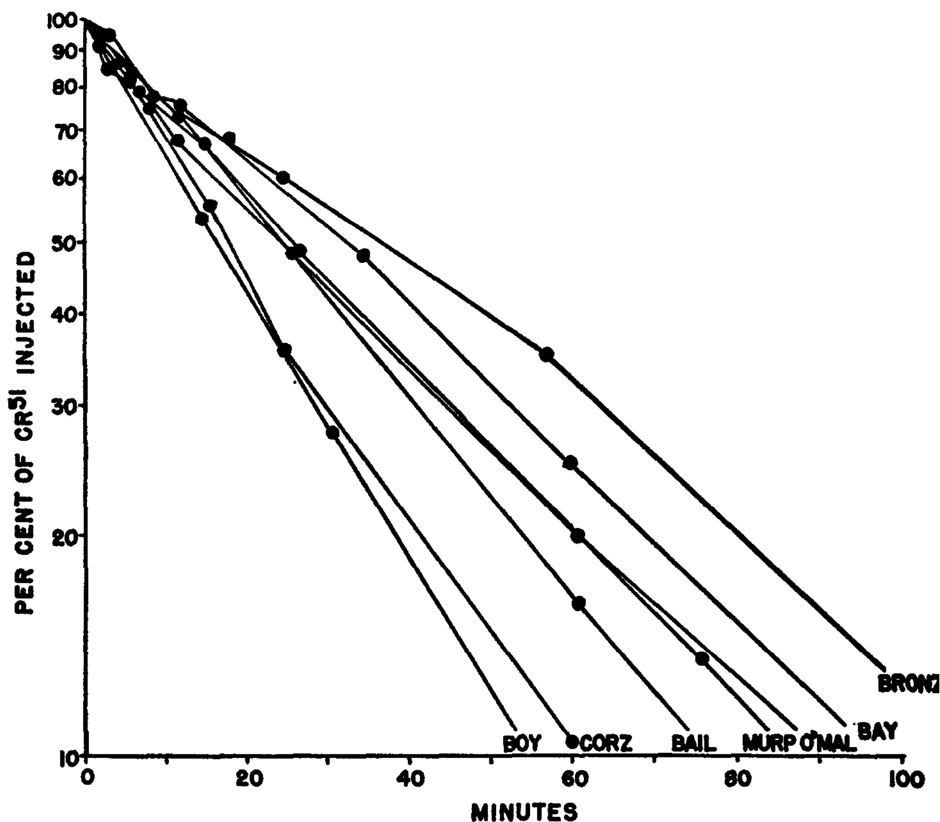

Fig. 3. The survival in normal individuals of Cr^{51}-labeled red cells sensitized with various anti-Rh sera.

it was complicated by splenomegaly and leukopenia. In order to determine whether the alacrity with which sensitized red cells were sequestered by this patient was attributable to rheumatoid factor activity or to an abnormally avid reticuloendothelial system, cells were injected that had been altered by a non-immunologic mechanism. For this purpose, normal Cr^{51}-labeled cells that had been treated with 8 mM N-ethylmaleimide (NEM) (20) were injected; the half-survival of such cells in normal subjects is similar to that of Rh-sensitized cells, about 22 minutes (21). In patient Mar., however, their half-survival was

14 minutes. Consequently, we suspect that in this patient a hyperactive reticuloendothelial system is primarily responsible for the abnormally rapid clearance of sensitized red cells.

In order that the patients' rheumatoid factors had adequate opportunity to

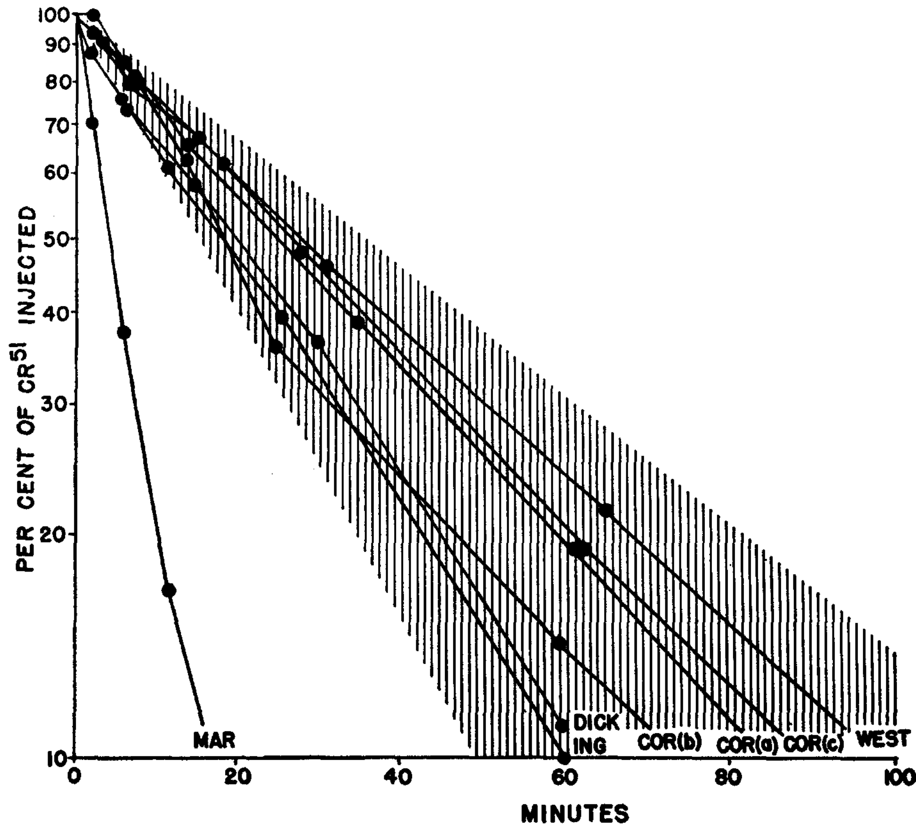

Fig. 4. The survival in patients with rheumatoid arthritis of Cr^{51}-labeled red cells sensitized with various anti-Rh sera. The shaded area represents the range of survival of similarly sensitized cells in normal individuals (Fig. 3).

react with the injected, sensitized red cells, these cells on two occasions were incubated *in vitro* with undiluted rheumatoid serum for 1 hour and thereafter infused into the rheumatoid serum donor. At the completion of the incubation period there was some rouleaux formation but little agglutination. The kinetics of red cell sequestration were not accelerated by this preincubation procedure.

B. Heterologous Antiglobulins.—

The effect of an immune antiglobulin on the destruction of Rh-sensitized red cells was studied in 2 normal subjects and in 1 patient with rheumatoid

arthritis. Each of these subjects had been injected previously with sensitized red cells and had sequestered them normally. When such Cr^{51}-labeled sensitized red cells were admixed with 10 volumes of immunologically inert red cells and then incubated with sterile rabbit antiserum, approximately 10 per cent of the cells were agglutinated—an appropriate fraction considering the dilution of sensitized with non-sensitized red cells. After infusion, the sensitized cells disappeared from the circulation very promptly (Table VII, Fig. 5). Hepatic Cr^{51} uptake was prominent in 2 of the 3 cases; in the third subject progressively increasing precordial Cr^{51} activity suggested possible sequestration of some of the agglutinates in the pulmonary vascular bed.

DISCUSSION

The available evidence suggests that rheumatoid factors are antibodies reactive with 7S γ-globulins. The manifest ability of these macroglobulins:

TABLE VII

The Effect of Incubation with Rabbit Antiglobulin Serum on the Survival and Sequestration of Rh-Sensitized Red Cells

Subject	Anti-Rh	Cr^{51}-survival $t\ \frac{1}{2}$, min.	c.p.m. Spleen/liver
Bay.	Ges.	6	1.5
O'Mal.	"	3	0.5
Cor.	"	5.5	1.6

(*a*) to agglutinate particles (22) or red cells (23) coated with 7S γ-globulin; (*b*) to precipitate aggregated γ-globulin (6) and to enhance its phagocytosis *in vitro* (24); and (*c*) to form 22S complexes with native, 7S γ-globulins *in vivo* (1, 2), demonstrates conclusively that the rheumatoid factors do interact with γ-globulin, although it does not indicate whether this interaction is immunologic in nature. Agglutination (25, 26) and opsonization (25–27) are not specific indications for an immune reaction, and it is evident that specific protein-protein interactions, as between hemoglobin and haptoglobin (28), and specific protein-cell interactions, as between transferrin and immature red cells (29), may represent non-immunologic events. The real bases for the belief that rheumatoid factors are antibodies are that they possess the physico-chemical and antigenic properties of antibodies and that they manifest specificity in their reactions with various 7S γ-globulins. In seeming conflict with this concept was the finding by Grubb (30) that the parenteral administration of Gm (a+) γ-globulin to rheumatoid patients whose sera manifested anti-Gm (a) activity *in vitro* resulted in no systemic or local anaphylactoid reactions and no detectable diminution in the half-life of the administered γ-globulin. Accordingly, Grubb (31) conceives of rheumatoid factor as a "heavy weight brother of

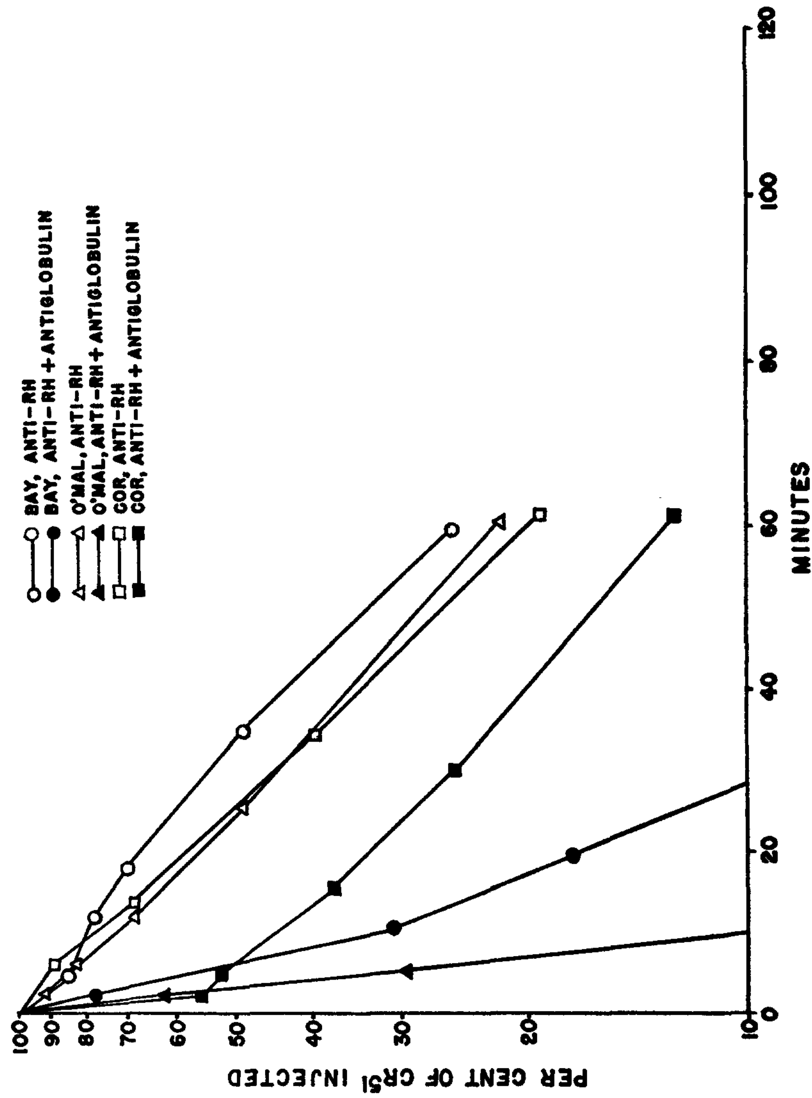

Fig. 5. The effect of preincubation with rabbit serum containing antibodies against human serum globulins on the survival in man of anti-Rh-sensitized red cells.

complement" and has pondered the question "... whether the addition of these macroglobulins ... to ... 7S antibodies might alter some function or manifestation of the 7S antibody". The present studies were designed to demonstrate whether rheumatoid factors can indeed modify antibody activity *in vivo* and, if so, to compare their effects with those of classical antiglobulin antibodies.

Initial experiments in the rat established that the interaction of immune rat anti-rabbit globulin with homologous red cells sensitized with incomplete rabbit antibody markedly accelerated the sequestration of the sensitized cells, primarily within the liver. It could be shown that quantities of coating rabbit antibody that alone were insufficient to provoke red cell sequestration *in vivo*, did so after addition of immune antiglobulin (Fig. 1 B). Analogous studies in man demonstrated that immune rabbit anti-globulin hastened the destruction of Rh-positive red cells coated with incomplete Rh antibody. Both in man and in the rat the intensity of red cell sequestration induced by immune antiglobulins directly paralleled their agglutinating activity with respect to the sensitized cells.

In contrast, the sequestration of sensitized red cells in patients with rheumatoid arthritis was not accelerated, except in a single patient with "hypersplenism," although serum for each was capable of agglutinating the sensitized cells *in vitro*. The absence of significant red cell agglutination when sensitized cells were mixed with undiluted rheumatoid serum (a prozone phenomenon) seemed the most likely explanation of this finding. In an attempt to circumvent the prozone, sensitized cells were agglutinated *in vitro* with dilute rheumatoid serum (Cor.) prior to their infusion. Despite this maneuver, the rate of destruction of these cells after their injection into the rheumatoid patient was not accelerated (Fig. 6). Presumably the agglutinates immediately dispersed *in vivo* much as they do *in vitro* in undiluted rheumatoid serum.

As previously stated, despite intensive efforts to locate a single rheumatoid serum that, when undiluted, strongly agglutinated Rh-sensitized red cells, we were unable to do so. Others have reported that such rheumatoid sera do exist (32–34) and that prozones, when found, may be ascribed to the simultaneous occurrence in the rheumatoid serum of an anti-Gm (a, b, or x) rheumatoid factor and the corresponding Gm-type of gamma globulins (32, 35). However, we do not believe that such reasoning can satisfactorily explain the prozones herein described. Harboe (34) has reported that red cells sensitized with either "Ripley" or "Murphy" are strongly agglutinated by nearly all rheumatoid sera and that "prozones are rare." It is difficult to believe that all 35 of our randomly selected rheumatoid sera would, on the basis of chance alone, agglutinate Ripley- or Murphy-sensitized red cells with the formation of prozones. Rather we suggest that this discrepancy may arise from differences in methodology. In the present studies (*a*) titrations were begun with whole serum; (*b*) care was taken to distinguish between the effect on sensitized cells

of rouleaux-forming globulins[11] and that of true agglutinins; and (*c*) observations were made on unspun suspensions incubated no longer than 2 hours. It is probable that procedures such as centrifugation or overnight storage of cell

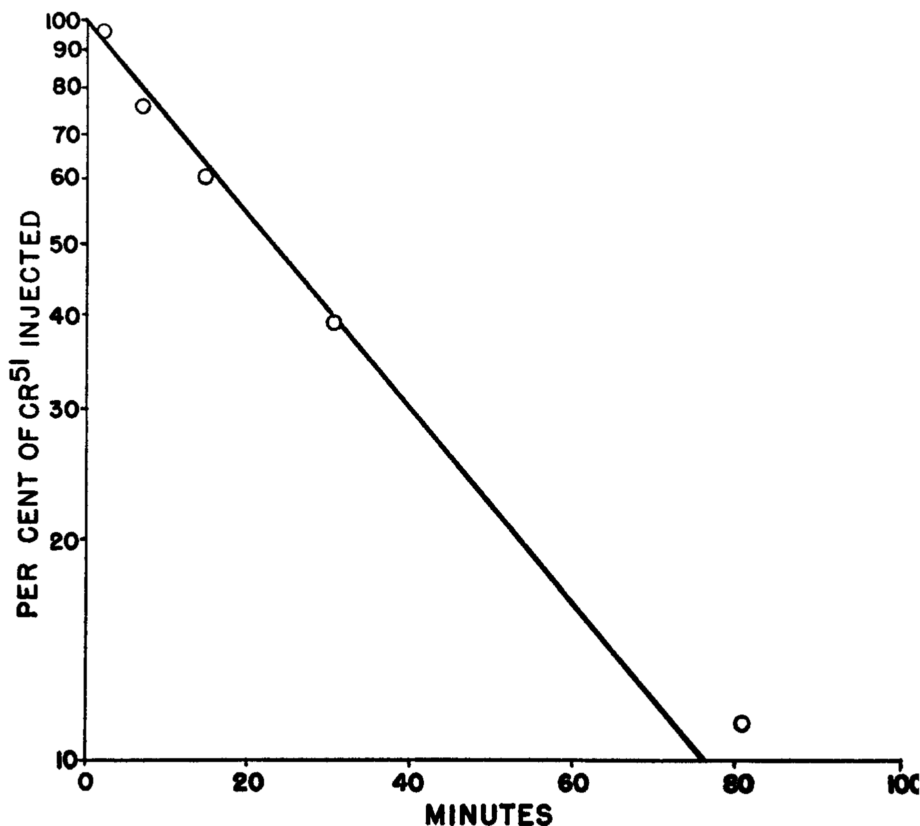

FIG. 6. The survival in patient Cor. of Cr^{51}-labeled Rh-sensitized red cells agglutinated, before injection, by dilute Cor. serum.

suspensions in the cold may yield different results that of necessity would bear less strongly on *in vivo* mechanisms. It is possible, of course, that rheumatoid patients do exist whose whole serum agglutinates Rh-sensitized red cells both *in vitro* and *in vivo*. Whether, in such individuals, significant alteration in the kinetics would occur is open to speculation. Mollison (37) noted an abnormally

[11] It should be noted whenever titering sera (including rheumatoid sera) that contain elevated concentrations of γ-globulin, that the first one or two dilutions may contain sufficient globulin levels to agglutinate sensitized cells non-specifically through the induction of rouleaux formation (36). Accordingly, the existence of a prozone may be more readily seen in serum dilutions between 1:2 and 1:4.

rapid disappearance of Rh-sensitized red cells in one patient with severe rheumatoid arthritis. Serum from this patient agglutinated the sensitized cells *in vitro*, and this activity was attributed to the presence of "Grubb type of agglutinating factor." However, there is no information on the size of the patient's spleen, the primary site of sequstration of the sensitized red cells or the effect of the patient's undiluted serum on the sensitized cells.

In order to explore the interaction of rheumatoid factor with incomplete coating antibody in the prozone region, a variety of *in vitro* studies were performed. As previously reported by Grubb (30) it was found that simple dilution with saline of a prozone system produced prompt red cell agglutination. An analogous volume effect in the F-II precipitin reaction has been observed by Vaughan and others (38). This latter phenomenon was attributed by Christian (39) and by Vaughan (40) to an inhibitory effect of the 7S γ-globulin present. The induction of agglutination by the simple addition of saline indicates that it is the relative concentration, rather than the absolute quantity, of γ-globulin present that must determine the degree of inhibition produced. If, as has been shown by others (39, 41), the reactivity of rheumatoid macroglobulin with 7S γ-globulin is related to the degree of aggregation of the γ-globulin (*i.e.* the concentration or density of γ-globulin molecules at a given locus) a theory may be constructed to explain the observed volume effects. Free γ-globulin molecules in the plasma and those sterically fixed or aggregated by physical (*e.g.* heating) or immunological means may compete for combining sites on the rheumatoid macroglobulin. In an undiluted system, free γ-globulin is present in relatively high concentration and can compete successfully for rheumatoid factor. The latter is, thereby, prevented from reacting extensively with the lesser amounts of aggregated or cell-fixed γ-globulin and suboptimal precipitation or agglutination results. In more dilute systems the concentration of free γ-globulin is diminished; however, no concomitant reduction in the concentration of aggregated γ-globulin or of the γ-globulin fixed at the cell-medium interface can occur. The interaction of rheumatoid macroglobulin with the fixed γ-globulin is thereby favored and results in augmented precipitation or agglutination.

Viewed in this context it is understandable why rheumatoid factors are not absorbed from undiluted rheumatoid serum by packed, sensitized red cells. The maximum quantity of specific antibody that can be affixed immunologically to the surface of a red cell is limited by the availability of the corresponding antigen (42). On the addition of undiluted rheumatoid serum to optimally sensitized cells, the density of free γ-globulin may greatly exceed that of the aggregated antibody fixed at the cell-serum interface. Consequently, there is little tendency for rheumatoid macroglobulin to interact with the sensitized cells. If, however, one attempts to elute rheumatoid factor from such cells, they must first be washed in saline. The addition of saline necessarily dilutes the free γ-globulin present. The γ-globulin density at the cell surface

may then rival that of the medium, favoring union with rheumatoid factor and, thereby, cell agglutination. Spuriously large quantities of rheumatoid factor activity may then be eluted from the washed cells by standard techniques. Such a phenomenon may account for the results of many reported absorption studies (43, 44).

The interpretation offered above does not conflict with the view that rheumatoid factors are antibodies directed against γ-globulin. The results are also compatible with the concept that rheumatoid factors are weakly reactive with all unaltered γ-globulin (9) and more strongly reactive with buried antigenic determinants in the γ-globulin molecule that are exposed during molecular fixation or aggregation.

Whatever the nature of the bonds between rheumatoid factors and undenatured γ-globulins, it is apparent that they are extraordinarily labile or, at least, readily reversible. That this is so is indicated by the fact that sensitized red cells that have been agglutinated by dilute rheumatoid serum, are immediately disagglutinated when the same serum is added thereafter in undiluted form. Accordingly, it is possible that the stability of the bonding is critically dependent on the number of combining sites involved and that only when the local concentration of reacting γ-globulin sites is sufficiently great to permit multiple interactions with each macroglobulin molecule does agglutination occur. Thus, an anti-Rh γ-globulin such as that of Ripley that possesses multiple Gm groups would, and does, react more strongly than an ordinary anti-Rh globulin. An anti-Rh sera such as Murphy, containing antibodies to more than one factor at the Rh locus could create a clustering of γ-globulins at the cell surface that would favor multiple reactions with a rheumatoid factor. Finally, aggregated γ-globulin would have a marked advantage by virtue of its high density of fixed γ-globulin sites, and indeed, aggregated γ-globulin is actually precipitable by whole, undiluted rheumatoid serum *in vitro* (1, 39, 41).

Whatever hypothesis is advanced, the present studies indicate that in the usual patient with rheumatoid arthritis there exists a combination of factors that effectively prevents the interaction of rheumatoid macroglobulin with incomplete Rh antibodies, whether or not there exists demonstrable Gm "incompatibility." The possibility must be recognized that other, more highly aggregated, antigen-antibody complexes may react *in vivo* with rheumatoid macroglobulins much more efficiently, and, perhaps with pathologic consequence (41). To date there have been no reported studies *in vivo* bearing on this concept.

If rheumatoid macroglobulins are indeed capable of effecting pathologic damage *in vivo*, the latter should logically first become manifest in a hypogammaglobulinemic environment. It is significant that rheumatoid arthritis is primarily a disease of synovial membranes (45). Synovial fluids being normally hypoproteinemic (46) may, as a consequence, be a perfect milieu to

activate the postulated deleterious properties of rheumatoid factors. In this context the hypergammaglobulinemia that commonly occurs in rheumatoid patients may be considered as "protective." Admittedly these hypotheses are highly conjectural; however, they are of sufficient theoretic interest to merit further investigation.

SUMMARY

Studies were undertaken in man and in the rat comparing the effects of rheumatoid factors and immune antiglobulins on red cells sensitized with incomplete antibodies.

The interaction of immune antiglobulins with sensitized red cells produced (*a*) agglutination *in vitro* and (*b*) an accelerated sequestration of the sensitized cells *in vivo*. In contrast, rheumatoid macroglobulins, although capable of agglutinating Rh-sensitized red cells *in vitro*, did not modify their destruction *in vivo*. The failure of rheumatoid factors to function as antiglobulins *in vivo* appears to reflect their non-reactivity with sensitized cells in whole serum.

It is suggested: (*a*) that the native (7S) gamma globulins of plasma competitively inhibit rheumatoid factors from reacting with fixed antibody in the blood stream; (*b*) that if these macroglobulins do indeed have pathogenetic activity, this may be limited to body fluids of low protein content.

The technical assistance of Laurie T. Dancy is gratefully acknowledged.

BIBLIOGRAPHY

1. Franklin, E. C., Holman, H. R., Müller-Eberhard, H. J., and Kunkel, H. G., An unusual protein component of high molecular weight in the serum of certain patients with rheumatoid arthritis, *J. Exp. Med.*, 1957, **105,** 425.
2. Franklin, E. C., Kunkel, H. G., Müller-Eberhard, H. J., and Holman, H. R., Relation of high molecular weight proteins to the serological reactions in rheumatoid arthritis, *Ann. Rheum. Dis.*, 1957, **16,** 315.
3. Rose, H. M., Ragan, C., Pearce, E., and Lipman, M. O., Differential agglutination of normal and sensitized sheep erythrocytes by sera of patients with rheumatoid arthritis, *Proc. Soc. Exp. Biol. and Med.*, 1958, **68,** 1.
4. Waller, M. V., and Vaughan, J. H., Use of anti-Rh sera for demonstrating agglutination activating factor in rheumatoid arthritis, *Proc. Soc. Exp. Biol. and Med.*, 1956, **92,** 198.
5. Grubb, R., Agglutination of erythrocytes coated with "incomplete" anti-Rh by certain rheumatoid arthritis sera on some other sera, *Acta Path. et Microbiol. Scand.*, 1957, **39,** 195.
6. Epstein, W., Johnson, A. M., and Ragan, C., Observations of a precipitin reaction between serum of patients with rheumatoid arthritis and a preparation (Cohn fraction II) of human gamma globulin, *Proc. Soc. Exp. Biol. and Med.*, 1956, **91,** 235.
7. Foz, A., Batalla, E., and Espacio, Y. L., "Autoantibodies" in rheumatoid arthritis

serum. A simple method for their demonstration with eventual diagnostic interest, *Rev. Diagn. Biol. (Madrid)*, 1954, **3,** 460.
8. Kunkel, H. G., Significance of the rheumatoid factor, *Arthritis and Rheumatism*, 1958, **1,** 381.
9. Fudenberg, H. H., and Kunkel, H. G., Specificity of the reaction between rheumatoid factors and gamma globulin, *J. Exp. Med.*, 1961, **114,** 257.
10. Vaughan, J. H., and Butler, V. P., Current status of the rheumatoid factor, *Ann. Int. Med.*, 1962, **56,** 1.
11. Harris, J., and Vaughan, J. H., Transfusion studies in rheumatoid arthritis, *Arthritis and Rheumatism*, 1961, **4,** 47.
12. Kaplan, M. E., and Jandl, J. H., Rheumatoid factors, antiglobulins and immune hemolysis, *Clin. Research*, 1962, **10,** 186 (abstract).
13. Jandl, J. H., and Kaplan, M. E., The destruction of red cells by antibodies in man. III. Quantitative factors influencing patterns of hemolysis *in vivo*, *J. Clin. Inv.*, 1960, **39,** 1145.
14. Kaplan, M. E., and Jandl, J. H., Inhibition of red cell sequestration by cortisone, *J. Exp. Med.*, 1961, **114,** 921.
15. Emerson, C. P., Franklin, W., and Lowell, F. C., The production of potent antihuman globulin (Coombs reagent) in rabbits immunized with serum adjuvant mixtures, *J. Immunol.*, 1951, **66,** 323.
16. Jandl, J. H., Greenberg, M. S., Yonemoto, R. H., and Castle, W. B., Clinical determination of the sites of red cell sequestration in hemolytic anemias, *J. Clin. Inv.*, 1956, **35,** 382.
17. Jandl, J. H., Jones, A. R., and Castle, W. B., The destruction of red cells by antibodies in man. I. Observations on the sequestration and lysis of red cells altered by immune mechanisms, *J. Clin. Inv.*, 1957, **36,** 1428.
18. Grubb, R., and Laurell, A. B., Hereditary serological human serum groups, *Acta Path. et Microbiol. Scand.*, 1956, **39,** 390.
19. van Loghem, J. J., Kresner, M., Coombs, R. R. A., and Fulton Roberts, G., Observations on a prozone phenomenon encountered in using the anti-globulin sensitization test, *Lancet*, 1950, **2,** 729.
20. Jacob, H. S., and Jandl, J. H., Effects of sulfhydryl inhibition on red blood cells. I. Mechanism of hemolysis, *J. Clin. Inv.*, 1962, **41,** 779.
21. Jacob, H. S., and Jandl, J. H., Effects of sulfhydryl inhibition on red blood cells. II. Studies *in vivo*, *J. Clin. Inv.*, 1962, **41,** 1514.
22. Singer, J. M., and Plotz, C. M., The latex fixation Test. I. Application to the serologic diagnosis of rheumatoid arthritis, *Am. J. Med.*, 1956, **21,** 88.
23. Ziff, M., The agglutination reaction in rheumatoid arthritis, *J. Chron. Dis.*, 1957, **5,** 644.
24. Parker, R. L., and Schmid, F. R., Phagocytosis of particulate complexes of gamma globulin and rheumatoid factor, *J. Immunol.*, 1962, **88,** 519.
25. Landsteiner, K., and Jagič, N., Ueber Reacktionen Anorganischer Kolloide und Immunkörperreaktionen, *Münch med. Woch.*, 1904, **51,** 1185.
26. Jandl, J. H., and Simmons, R. L., The agglutination and sensitization of red cells by metallic cations: Interactions between multivalent metals and the red cell membrane, *Brit. J. Haematol.*, 1957, **3,** 19.

27. Neufeld, F., and Etinger-Tulczynska, R., Beitrag zur Wirkungsweise der Phagozytoseerregenden Immunkörper, *Zentr. Bakt. 1, Abt.*, 1929, **114,** 252.
28. Laurell, C. B., and Nyman, M., Studies on the serum haptoglobin level in hemoglobinemia and its influence on renal excretion of hemoglobin, *Blood*, 1957, **12,** 493.
29. Jandl, J. H., and Katz, J. H., The plasma-to-cell cycle of transferrin in iron utilization, *Tr. Assn. Am. Physn.*, 1961, **74,** 72.
30. Grubb, R., Interactions between rheumatoid arthritic sera and human gamma globulin, *Acta Haematol.*, 1958, **30,** 246.
31. Grubb, R., The Gm groups and their relation to rheumatoid arthritis serology, *Arthritis and Rheumatism*, 1961, **4,** 195.
32. Harboe, M., Relation between Gm types and hemagglutinating substances in rheumatoid sera, *Acta Path. et Microbiol. Scand.*, 1960, **50,** 89.
33. Harboe, M., Simultaneous occurrence of hemagglutinating substances of different specificities in rheumatoid sera, *Acta Path. et Microbiol. Scand.*, 1960, **49,** 381.
34. Harboe, M., Interactions between red cells coated with incomplete anti-D and rheumatoid sera, *Acta Path. et Microbiol. Scand.*, 1960, **50,** 383.
35. Swahn, B., and Grubb, R., Purification of hemagglutinating factors in rheumatoid arthritic sera by Cohn's method 10 and ultracentrifugation, *Acta Path. et Microbiol. Scand.*, 1958, **42,** 173.
36. Jandl, J. H., and Castle, W. B., Agglutination of sensitized red cells by large anisometric molecules, *J. Lab. and Clin. Med.*, 1956, **47,** 667.
37. Mollison, P. L., Further studies on the removal of incompatible red cells from the circulation, *Proc. 7th Internat. Soc. Blood Transfusion*, 1959, 495.
38. Vaughan, J. A., Ellis, P. J., and Marshall, H., Quantitative considerations of the rheumatoid factor, *J. Immunol*, 1958, **81,** 261.
39. Christian, C. L., Characterization of the "reactant" (gamma globulin factor) in the F II precipitin reaction and the F II tanned sheep cell agglutination test, *J. Exp. Med.*, 1958, **108,** 139.
40. Vaughan, J. H., and Waller, M. V., Specificity of the rheumatoid factor, *Arthritis and Rheumatism*, 1958, **1,** 262 (abstract).
41. Edelman, G. M., Kunkel, H. G., and Franklin, E. C., Interaction of the rheumatoid factor with antigen-antibody complexes and aggregated gamma globulin, *J. Exp. Med.*, 1958, **108,** 105.
42. Masouredis, S. P., Reaction of I^{131} trace labeled human anti Rh_0 (D) with red cells, *J. Clin. Inv.*, 1959, **38,** 279.
43. Harboe, M., Interactions of rheumatoid factor with immune precipitate containing antibody of human origin, *Ann. Rheum. Dis.*, 1961, **20,** 363.
44. Aho, K., Kirpilä, J., Wager, O., and Virkkunen, M., Reaction of the rheumatoid factor with human specific precipitate, *Ann. Med. Exp. et Biol. Fenniae*, 1961, **39,** 66.
45. Cecil, R. L., and Loeb, R. F., Textbook of Medicine, Philadelphia, W. B. Saunders & Co., 1959.
46. Ropes, M. W., and Bauer, W., Synovial Fluid Changes in Joint Disease, Cambridge, Harvard University Press, 1953.