STRUCTURAL ASPECTS OF HUMAN ERYTHROCYTE AUTOANTIBODIES*

I. L Chain Types and Electrophoretic Dispersion

By JOHN P. LEDDY, M.D., and RICHARD F. BAKEMEIER,‡ M.D.

(From the Department of Medicine, University of Rochester School of Medicine and Dentistry, Rochester, New York)

(Received for publication, July 24, 1964)

In many cases of "autoimmune" hemolytic disease (AHD) the patient's red cells (RBC) are sensitized with autologous 7S γ-globulin[1] (see reference 2). In an effort to gain some insight into the pathogenesis of these autoantibodies, studies on their structural homogeneity or heterogeneity have been undertaken. Numerous investigations of the homogeneous γ-globulins produced by plasmacytic tumors (myeloma) in man and mice (see reference 3) have pointed to the possibility that structural homogeneity of a given population of γ-globulin molecules may reflect some degree of homogeneity of the cell population synthesizing those molecules. Similar structural studies of erythrocyte autoantibodies may contribute to the eventual characterization of their cells of origin.

The present investigation made use of a simple antigenic marker system. Human 7S γ-globulins appear to be composed of two pairs of dissimilar polypeptide chains, termed "heavy" (H) chains and "light" (L) chains because of differing molecular weights (4, 5). The L chains have been found to be of two major antigenic types, type I and type II, based on their abilities to elicit specific antibodies in rabbits (6–10). Evidence has been adduced that a given γ-globulin molecule carries L chains of either type I *or* type II (9, 10). The serum 7S γ-globulins of healthy donors have been found to contain molecules having type I or type II L chains in roughly a 2:1 ratio, respectively (9, 10). A given lymphoid cell appears to synthesize either type I or type II γ-globulins, or perhaps both types very rarely (11). Isolated antibodies to a given antigen made by a normal individual have been reported to include molecules of both L chain types, although the ratio of type I to type II molecules may deviate greatly from the 2:1 ratio in the individual's whole serum (12, 13). By contrast, myeloma globulins in the serum

* Presented in part before the American Society for Clinical Investigation May 3, 1964 (1). Supported by a grant from the National Foundation and by Public Health Service grant AI-04841 from the National Institutes of Health, United States Public Health Service.

‡ United States Public Health Service Research Career Development Awardee.

[1] These autoreactive γ-globulins will subsequently be termed "erythrocyte autoantibodies" for ease of reference.

of a given patient are composed exclusively of molecules having either type I *or* type II L chains (7, 8).

This report will present evidence that populations of isolated erythrocyte autoantibodies exhibit a strikingly high frequency of L chain homogeneity. Some of these autoantibody populations bearing only one detectable L chain type may also display relative homogeneity in electrophoretic mobility.

Materials and Methods

Human Antibodies.—For isolation of autoantibodies, bleedings from patients[2] were taken into 0.01 M Na_2EDTA or ACD solution.[3] Eluates from 6 times washed RBC stromata were made by acidification (14) or by heating at 60°C for 30 minutes.[3] Anti-Rh_o antibodies (anti-D) (anti-Rh1) (15) from individual donor sera[4] were reacted at 37°C with the 6 times washed RBC of a healthy donor whose phenotype was O Rh_o (ccDee) (Rh: 1, −2, −3, 4, 5) (15). Eluates from the washed, sensitized RBC were made by acidification. These eluates displayed no reactivity with O rh (ccddee) (Rh: −1, −2, −3, 4, 5) RBC. None of the Rh antisera or eluates contained demonstrable saline agglutinating activity for O Rh_o RBC. Autoantibody and Rh antibody eluates were concentrated by negative pressure ultrafiltration through collodion membranes. The only immunoglobulin detectable by hemagglutination and immunodiffusion in the concentrated eluates was 7S γ-globulin.[5]

Antigens.—Bence Jones (BJ) proteins, the pathologic equivalent of normal L chains (16), were isolated from urines of myeloma patients, Sm. (type I) and Sl. (type II), by precipitation in 55 per cent saturated ammonium sulfate followed by chromatography on DEAE cellulose (17). Highly purified 7S myeloma globulins were obtained by utilizing their properties as euglobulins or cryoglobulins. The original typing of BJ and myeloma proteins was established by Ouchterlony analyses employing reference BJ proteins kindly supplied by Dr. Leonhard Korngold (6).

Antisera.—Anti-Bence Jones sera (anti-BJ) were made in rabbits by immunization with the Sm. or Sl. BJ protein in complete Freund's adjuvant, followed by intravenous administration of alum precipitates. The resulting antisera were absorbed in small increments with BJ protein and isolated 7S myeloma globulin of the opposite type, and finally with the fast fragment of normal human 7S γ-globulin prepared by published methods (18, 19). In Ouchterlony analysis the absorbed antisera gave a single precipitin line with Cohn Fr. II, and this line fused completely with the lines given by several BJ proteins and myeloma globulins of the corresponding type and with the line given by L chains obtained from normal γ-globulin (20). No precipitin lines were detected against a wide range of concentrations of the following antigens: BJ proteins and myeloma globulins of the opposite type, the fast fragment from normal γ-globulin, and the urinary "H chains" of Franklin's patient, Cr. (21).[6] *Anti-7S*

[2] We are indebted to the following physicians for allowing us to study their patients: Dr. Scott N. Swisher, Dr. Stanley B. Troup, and Dr. James Carlin, Rochester, New York; Dr. John H. Crookston and Dr. Richard C. Hasselback, Toronto, Canada; Dr. Ben Fisher, Buffalo; Dr. Allyn Judd, Delray Beach, Florida; Dr. Russell Weisman, Jr. and Dr. Samuel Gross, Cleveland; Dr.Richard Platzer, Clifton Springs, New York; Dr. Nathan A. Cohen and Dr. Kenneth Ho, Syracuse, New York. We are further indebted to Dr. Swisher for the generous donation of previously made eluates on patients St., Th., Se., Ka., Tr.(a), and Coo.

[3] Collection of blood in acid citrate dextrose solution (ACD) and elution by heating were the procedures used in the preparation of the eluates obtained from Dr. Swisher.

[4] Two of these anti-Rh_o sera were kindly donated by Dr. John H. Crookston.

[5] Synonyms of 7S γ-globulin are γ_2-globulin and γ_{ss}-globulin.

[6] We are grateful to Dr. E. C. Franklin for supplying this protein.

γ-globulin (anti-7S), prepared by intravenous immunization of rabbits with Cohn Fr. $II_{1,2}$ precipitated in alum, contained precipitating antibodies to the H chains of 7S γ-globulin and to both types of L chains. *Antiserum to the H chains of 7S γ-globulin (anti-Hγ₂)* was prepared by absorbing anti-7S serum with isolated normal human L chains (20). This antiserum reacted with the H chain portions of both fast and slow fragments of human 7S γ-globulin. Antisera specific for the fast fragment of 7S γ-globulin (anti-Fγ₂), for γ_1A (β_2A)-globulin, for γ_1M

TABLE I

Specificity of the Anti-BJ Sera in Hemagglutination

RBC sensitized by	Antiserum used in antiglobulin reaction	Resulting agglutination (titer)
Ri. anti-Rh_o serum	Anti-BJ_I + saline	>160
	Anti-BJ_I + L chains*	0‡
	Anti-BJ_I + BJ_I§	0
	Anti-BJ_I + BJ_{II}§	>160
	Anti-BJ_I + H chains§	>160
Ri. anti-Rh_o serum	Anti-BJ_{II} + saline	>160
	Anti-BJ_{II} + L chains*	0
	Anti-BJ_{II} + BJ_I§	>160
	Anti-BJ_{II} + BJ_{II}§	0
	Anti-BJ_{II} + H chains§	>160
BDB–myeloma I‖	Anti-BJ_I	80
	Anti-BJ_{II}	0
	Anti-7S	1280
BDB–myeloma II‖	Anti-BJ_I	0
	Anti-BJ_{II}	40–80
	Anti-7S	1280
BDB–normal 7S‖	Anti-BJ_I	++
	Anti-BJ_{II}	++
	Anti-7S	++++

* 40 μg N/ml final concentration.

‡ Zero (0) indicates no reaction with 1/10 dilution of antiserum.

§ 20 μg N/ml final concentration.

‖ 250 μg N/ml of isolated type I or II myeloma globulins, or Cohn Fr. II.

(β_2M)-globulin and for the β_1C-β_1A–globulin component of complement (anti-C′) have been described in detail elsewhere (22). All antisera were thoroughly absorbed with normal human RBC of each major blood group prior to use in antiglobulin reactions.

Confirmation of the specificity of each anti-BJ serum was obtained by: (*a*) PCA reactions (23) in which isolated type I and type II myeloma globulins were the challenging antigens; (*b*) antiglobulin reactions with RBC coated with either type I or type II myeloma globulin by bisdiazotized benzidine (*BDB*) (24); and (*c*) specific blocking of antiglobulin reactions against RBC sensitized by Rh antibody. The latter two methods are illustrated in Table I. Specific hemagglutination inhibition was produced by the homologous BJ protein or normal

L chains (20) but not by the opposite BJ protein or H chains (Cr.). With the BDB method each anti-BJ serum agglutinated only those RBC coated with the appropriate myeloma globulin.

Hemagglutination Methods.—Preliminary tests were carried out to determine the optimum dilution of the eluted, concentrated autoantibodies to effect maximum sensitization without producing non-specific clumping of RBC. Although the autoantibodies reacted with every donor cell tested, the group O rh (ccddee) RBC of a single healthy donor were chosen as the standard test cells. The group O Rh_o (ccDee) RBC previously mentioned were employed for testing anti-Rh_o antibodies. In all cases sensitization was carried out at 37°C for 1 hour, followed by antiglobulin (Coombs) testing, (see reference 25 for details). Genetically deleted RBC, with the phenotypes O -D-/-D- (Hendsbee) and A ---/--- (Mackall), were generously donated by Dr. Philip Levine. These RBC had been glycerolized and stored at −50°C. Reconstitution of the cells for indirect antiglobulin reactions was by the method of Weiner (26).

Ouchterlony Diffusion and Immunoelectrophoresis.—Radial double diffusion reactions were carried out on microscope slides using 1 per cent agar in pH 7.3 phosphate-buffered saline (27). Immunoelectrophoresis was also performed on microscope slides with 1 per cent agar in 0.03 M barbital, pH 8.2 (28).

Quantitative Measurement of Type I and Type II γ-Globulins.—Using the quantitative Oudin technique (29, 30), as modified by Dr. John H. Vaughan (31), 7S γ-globulins chromatographically isolated (17) from the sera of normal subjects and AHD patients were allowed to diffuse against the anti-BJ sera. The actual concentrations of a given γ-globulin type in unknown samples were derived from a standard plot of analogous values obtained with a human 7S γ-globulin pool (Cohn Fr. II, Squibb lot 1967, E. R. Squibb and Sons, New York) This pool was assumed to contain type I and type II molecules in a 2:1 ratio (9, 10).

RESULTS

The RBC of 20 patients with "autoimmune" hemolytic disease (AHD) gave moderate to strong *direct* antiglobulin (Coombs) reactions with an antiserum to whole 7S γ-globulin (anti-7S) and with an antiserum specific for the fast fragment of 7S γ-globulin (anti-$F\gamma_2$). The RBC of 9 of the 16 patients so tested were also agglutinated by an anti-C′ serum (anti-β_1C-A-globulin).[7] None of the RBC samples was agglutinated by specific anti-γ_1A-globulin or anti-γ_1M-globulin sera. The anti-BJ sera gave negative reactions or equivocal typing patterns with all RBC samples tested in these *direct* antiglobulin reactions, despite many strong reactions with the anti-7S and anti-$F\gamma_2$-sera.

L Chain Typing.—

Indirect antiglobulin reaction:

For comparison to the above results, RBC sensitized *in vitro* by a commercial antiRh_o (anti-D) serum were tested with the same rabbit antisera. Heavily sensitized RBC's were agglutinated not only by anti-7S and anti-$F\gamma_2$ sera, but also by both anti-BJ sera. Weaker sensitization with more diluted Rh antibody gave either no L chain typing, or a false type I "mono-

[7] The presence of bound complement (C′) components on the RBC of many of our AHD patients has not caused any evident difficulty in eluting the antibody or in subsequently typing its L chains.

typic" pattern. Therefore, in typing L chains of either iso- or autoantibodies by hemagglutination, we relied upon *in vitro* sensitization of normal RBC, the intensity of which could be controlled and made fairly uniform as judged from hemagglutination results with anti-7S serum. Every effort was made to sensitize the test cells maximally, short of causing non-specific agglutinability.

When normal RBC were heavily sensitized *in vitro* with the autoantibody eluates from patients with AHD, a spectrum of reactivities with the anti-BJ sera was observed (Table II). The autoantibodies of 8 patients reacted only with anti-BJ_I (monotypic[8] type I). Those of 4 patients reacted only with anti-BJ_{II} (monotypic[8] type II). Those of 7 patients reacted with both antisera (bitypic). In the latter group, the eluates of El., Be., and No. showed very little reactivity with anti-BJ_{II}, while the eluate of He. showed only slight reactivity with anti-BJ_I.

In addition to the 19 autoantibody eluates just described, 2 eluates from another AHD patient, Tr., separated by an interval of more than 9 years, were of special interest. The earlier eluate, representing a concentrated pool of several eluates obtained between 1950 and 1955, gave reactions with *both* anti-BJ sera, with the anti-BJ_I reaction predominating [Tr. (a), Table II]. A recent eluate, of approximately equal strength as judged from hemagglutination with anti-7S, reacted *only* with anti-BJ_I [Tr. (b), Table II].[9]

Also recorded in Table II are reactions of RBC sensitized by 6 anti-Rh_o (anti-D) sera from donors without known hematologic disease. In 5 cases agglutination was clearly positive with both anti-BJ sera. Red cells sensitized by Gu. serum, however, consistently failed to react with the anti-BJ_{II} serum, despite levels of RBC sensitization apparently comparable to those achieved with the other anti-Rh_o sera. When RBC were sensitized with *isolated* anti-Rh_o antibodies from donors Ri., Bl., Ja., and Gu., obtained by the same acid elution and concentration techniques used for the autoantibodies, the results were essentially the same as those observed with RBC sensitized by the native sera (Table II).

Anti-Rh_o antibodies were present in the serum of AHD patient Tr. because of a previous incompatible transfusion. Both in native serum and when isolated by elution, these isoantibodies gave a bitypic pattern, with type I predominating, at a time when only type I L chains could be identified in the patient's autoantibody eluate, Tr. (b). No free autoantibody was detectable in her serum in tests with O rh red cells.

[8] For convenience the term "monotypic" will be applied to eluates reacting with only one of the two anti-BJ sera. It is recognized, however, that these hemagglutination methods, although sensitive, might fail to detect a very minor population of molecules of the opposite type.

[9] Although the earlier Tr. eluates, Tr. (a), were made by heating, while the recent Tr. eluate, Tr. (b), was obtained by acidification, there is no apparent reason to attribute the differences in the Tr. eluates to the elution methods (*cf.* Discussion).

TABLE II

Summary of L Chain Typing by Hemagglutination

Normal RBC sensitized by	Antiglobulin reactions (titers)			Known duration of positive Coombs' test‡	Recognized associated disease
	Anti-BJ_I	Anti-BJ_{II}	Anti-7S*		
AHD autoantibodies					
Fr. (55 M)§	320	0‖	5120	21 mos.	None
Re. (57 M)	320	0	2560	4 yrs.	"
Mo. (79 M)	160–320	0	5120	9 mos.	CLL¶
Con. (68 F)	160	0	5120	1 "	"
St. (63 M)	160	0	2560	1 "	"
Ma. (66 M)	160	0	5120	1 "	"
Ka. (59 F)	160	0	2560	6 "	None
Th. (88 F)	80	0	5120	2 "	"
El. (19 M)	160	5	5120	6 yrs.	Hodgkin's
Be. (75 F)	160	5	5120	2 mos.	None
No. (30 F)	320	20	5120	6 yrs.	"
Tr. (53 F) (a) (1950–55)	640	80	5120	1–5 "	"
" " (b) (1964)	320	0	2560	14 "	"
Coo. (22 F)	160	160	2560	2 "	"
Me. (36 M)	320	320	2560	5 "	"
Po. (39 F)	40	320	5120	10 "	"
He. (25 M)	20	640	>5120	13 "	"
Se. (77 M)	0	>80	>1280	10 mos.	Ca. lung
Sa. (64 F)	0	160	2560	14 "	Lymphosarcoma
Ad. (73 M)	0	320	5120	2 "	None
De. (56 F)	0	640	>5120	6 yrs.	"
Rh isoantibodies					
Ri. serum	640	640	2560		
" eluate	>160	160	>160		
Bl. serum	320	640	5120		
" eluate	320	640	5120		
Ja. serum	320	80	5120		
" eluate	640	40	5120		
Gu. serum	160	0	5120		
" eluate	640	0	5120		
Ro. serum	640	80	5120		
Gi. "	320	80–160	5120		
Tr. " (1964)**	160	40	2560		
" eluate (")	320	40	5120		

* Anti-Fγ_2 gave very comparable results, not shown.

‡ Duration prior to bleeding for isolation of autoantibodies.

§ (Age at onset, sex).

‖ Zero (0) indicates no reaction with 1/5 dilution of antiserum.

¶ CLL, chronic lymphocytic leukemia.

** AHD patient Tr.

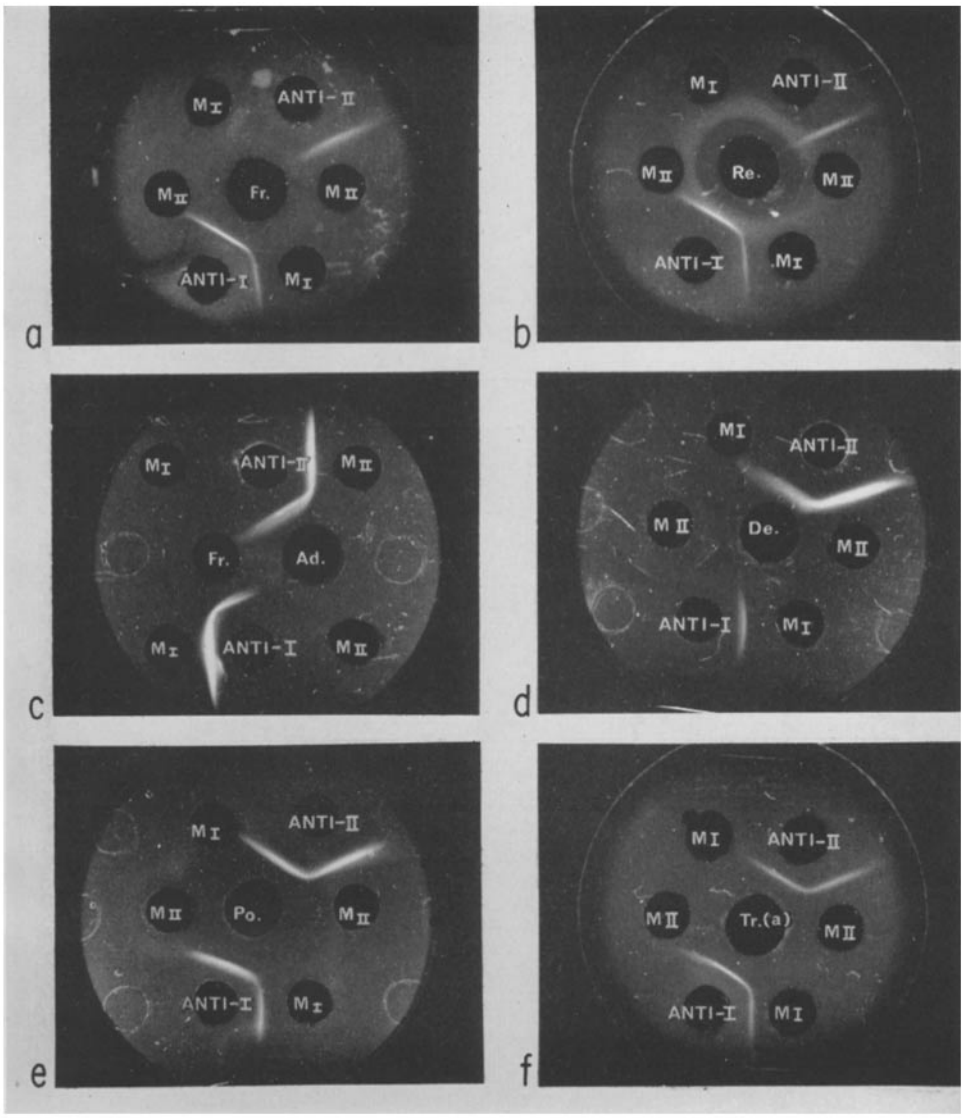

FIG. 1. Double diffusion (Ouchterlony) analyses of isolated erythrocyte autoantibodies. Center wells: Fr., Re., Ad., De., Po., and Tr.(a) concentrated eluates. *Anti-I*, anti-BJ_I; *anti-II*, anti-BJ_{II}. M_I, M_{II}; isolated type I and type II myeloma globulins, respectively.

Gel precipitin reactions: By means of Ouchterlony gel precipitin reactions the L chain typing was confirmed for those 7 autoantibody eluates having sufficient γ-globulin concentration to permit this analysis (Fig. 1). For example, the eluates of patients Fr. (Fig. 1 *a* and *c*), Re. (Fig. 1 *b*) and Mo. (not shown)

gave a precipitin line only with anti-BJ_I, and this line fused completely with that of a known type I myeloma globulin. With the eluates of patients Ad. (Fig. 1 *c*) and De. (Fig. 1 *d*) a precipitin line was produced only by anti-BJ_{II}, fusing with the line of a known type II myeloma globulin. In contrast, the autoantibodies of patient Po. (Fig. 1 *e*) and Tr. (a) (Fig. 1 *f*) gave strong precipitin lines with both antisera. Isolated Rh antibodies from donors Ri. and Bl. also gave precipitin reactions with both antisera (not shown).

TABLE III

Type I and Type II Serum 7S γ-Globulins from AHD Patients and Normal Donors

Patient (autoantibody type)	7S γ-globulin (μg N/ml of serum fraction)			
	Total*	Type I‡	Type II‡	Type I/Type II
Fr. (I)	188	132	83	1.58
Re. (I)	102	68	41	1.67
Con. (I)	95	57	39	1.44
De. (II)	110	73	42	1.73
Se. (II)	95	73	36	2.05
Sa. (II)	118	73	56	1.32
Ad. (II)	108	85	38	2.22
Po. (II > I)	133	84	44	1.89
Tr. (a) (I > II)	97	71	38	1.89
Tr. (b) (I)	84	61	30	2.05
10 Normals, Mean (±2 SD)	110	80	40	2.00 (±0.76)

* Analyzed with anti-$H\gamma_2$ serum (see Materials and Methods).

‡ Analyzed with corresponding anti-BJ serum.

Overall Production of Type I and Type II 7S γ-Globulins.—The quantitative Oudin technique was used to determine the relative proportions of type I and type II molecules in 7S γ-globulin fractions from the sera of 10 AHD patients and 10 normal persons. Despite the restricted representation of L chain types in many of the autoantibody populations, all of the AHD patients showed ratios of type I:type II 7S γ-globulins which fell within two standard deviations of the normal mean (Table III).

Autoantibody Specificity.—Because the inclusion of multiple combining specificities in a given autoantibody population might contribute to heterogeneity in its L chain types (see Discussion), selected testing of 12 eluates was carried out with genetically "deleted" RBC (32, 33). From Table IV it is evident that differences in specificity among these autoantibodies can be detected. One eluate (De.) reacted with normal RBC but not with either deleted cell. Three other eluates reacted with normal and -D-/-D- (partially deleted) cells, but

not with the ---/--- (fully deleted) cells. Many reacted with all 3 test cells. The last pattern has been shown (33) to result from the presence of antibodies reactive with 1, 2, or all 3 of the test cells, as determined by the capacity of each cell type to absorb all or part of the total reactivity of the eluate. After absorption with ---/--- cells neither the monotypic eluate of Ad. nor the bitypic eluate of Po. showed any residual reactivity with the other test cells (Table IV). The supply of deleted cells was insufficient for further absorption studies.

TABLE IV

Antiglobulin Reactions of Deleted RBC Sensitized by Autoantibodies

Eluate from patient:	L chain(s) detected	RBC used for testing		
		Normal	-D-/-D-	---/---
Fr.	I	++	+++	++
Re.	I	++	+	+++
Mo.	I	++	+	+
Con.	I	+++	+++	0
De.	II	+++	0	0
Sa.	II	++	++	0
Ad.	II	+++	+++	+++
Po.	II > I	+++	+++	+++
Coo.	I ≅ II	+++	++	+++
Tr. (a)	I > II	+++	+++	+++
No.	I ≫ II	+++	+++	+++
El.	I ≫ II	+++	++	0
Ad. absorbed*		0	0	0
Po. absorbed*		0	0	0

* Eluate, in suitable dilution, absorbed once at 37°C with 1/3 volume of packed ---/--- RBC. Absorptions were also carried out with -D-/-D- RBC, with similar results.

Electrophoretic Dispersion.—It was of interest to determine whether autoantibody populations showing only one L chain type would also be relatively homogeneous in their physical properties as compared to bitypic autoantibodies and isoantibodies. In Fig. 2 are shown immunoelectrophoretic patterns given by highly concentrated preparations of these antibodies with anti-7S serum. The autoantibody populations of patients Fr. (monotypic type I) and Ad. (monotypic type II) were relatively homogeneous electrophoretically (Fig. 2 *a* to *c*), closely resembling patterns observed with myeloma globulins (Fig. 2 *b*). On the other hand, the type I eluates of patients Re. and Tr. (b) showed greater electrophoretic dispersion (Fig. 2 *d* and *e*) approaching, but not quite equaling, that of normal 7S γ-globulins. The latter pattern was also observed

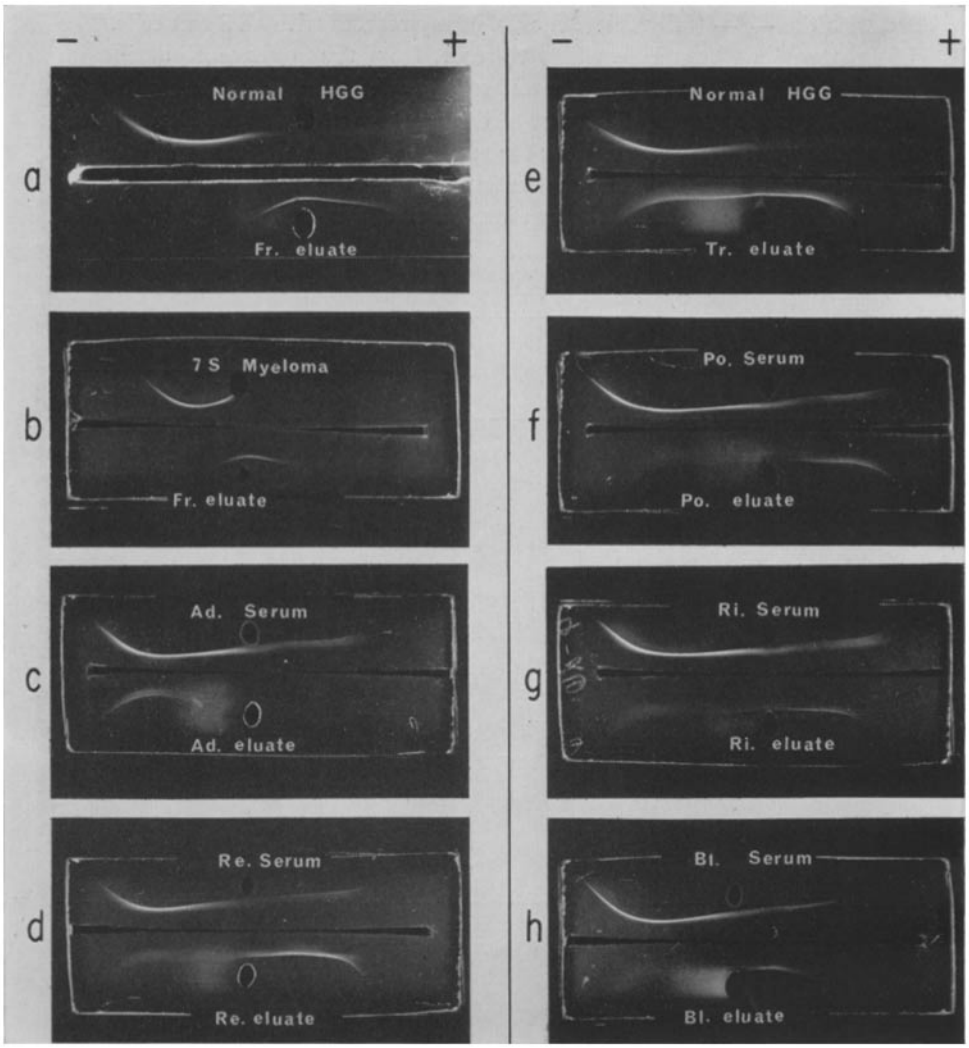

FIG. 2. Immunoelectrophoretic behavior of isolated autoantibodies and Rh antibodies. (*a*) and (*b*): Autoantibodies of patient Fr. compared to Cohn Fr. II (HGG) and to isolated 7S myeloma globulin. (*c*) through (*f*): Autoantibodies of patients Ad., Re., Tr.(b) and Po. compared to autologous serum or to Cohn Fr. II. (*g*) and (*h*): Ri. and Bl. Rh antibodies compared to autologous serum. The patterns were developed with anti-7S serum. The cathodal extensions of the precipitin lines given by Po., Ri., and Bl. eluates were more clearly visualized on the original plates.

with the bitypic eluate of patient Po. (Fig. 2 *f*) and with the isolated Rh antibodies from donor Ri. (Fig. 2 *g*). Isolated Rh antibodies from Bl. may be somewhat less polydisperse (Fig. 2 *h*) than those from Ri. Insufficient monotypic Gu. anti-Rh_o eluate is available at present for electrophoresis.

DISCUSSION

L Chain Types.—Isolated populations of normal human antibodies (12, 13) and of several types of "autoantibodies" (12, 13, 34, 35) have generally been found to be composed of both type I and type II γ-globulins, often with moderate predominance of type I. This is in keeping with the normal predominance of this type in human serum (9, 10). In contrast, a majority of the erythrocyte autoantibody populations examined in the present study were found to be remarkably homogeneous in their L chain content, exhibiting, by the techniques employed, L chains of entirely one type (12 cases) or with a marked predominance of one type (4 additional cases). An especially striking contrast to the latter finding is provided by the clearly demonstrated L chain *heterogeneity* of 17 of 18 populations of 7S anti-Rh_o (anti-D) antibodies studied to date (references 12, 13 and Table II). In fact, the anti-Rh_o *isoantibodies* of AHD patient Tr. contained γ-globulins of both types, even though the *autoantibody* eluate prepared from the same bleeding, Tr. (b), displayed only type I L chains (Table II). The finding of 4 autoantibody populations apparently composed entirely of type II γ-globulins is particularly striking. Since roughly only one-third of the serum 7S γ-globulins of these patients are type II (Table III; references 9, 10), these autoantibodies fall within a minority population of the patients' total serum γ-globulins. Evidence for relative L chain homogeneity of erythrocyte autoantibodies, with type II predominating, has also been presented by Franklin and Fudenberg in 2 of 3 AHD patients studied by *direct* antiglobulin tests (13).

Erythrocyte autoantibodies are not unique in exhibiting apparently complete or relative L chain homogeneity. Pathologic cold hemagglutinins and certain rheumatoid factors have been found to contain a single L chain type (12, 13, 36). It must also be pointed out that certain normal antibody populations have displayed a marked predominance of type I or type II molecules, although both types were detected (12).

The restricted L chain representation found in many of the erythrocyte autoantibodies in this study cannot be ascribed to a more general inability to synthesize L chains of the opposite type or to incorporate them into 7S molecules (Table III). It appears likely, therefore, that autoantibody populations with a high degree of L chain homogeneity reflect some selective effect in their synthesis. Possibly they are produced by sharply restricted populations of antibody-forming cells. This would be consistent with recent evidence that,

with rare exceptions, a given lymphoid cell contains L chain determinants of only one type (11).

According to current concepts of antibody formation, one might conceive of monotypic antibody populations arising in at least four circumstances. One such circumstance might be exceptional structural simplicity of the antigenic determinant eliciting the antibody response, as in chemical haptens. Very little is known, however, about the structural nature of the RBC determinants with which these autoantibodies react. *Secondly*, monotypic antibody populations might occur during the earlier stages of immunization. The duration of known erythrocyte autosensitization prior to this study, in the patients with monotypic autoantibodies, varied from 1 month to several years (Table II). Noteworthy are patients Re. and De. whose autoantibody populations were monotypic despite durations of disease which were at least 4 and 6 years, respectively. Patient Tr. demonstrated an autoantibody population which was monotypic after a 14 year duration, whereas it had been bitypic during the earlier years of her disease. *Thirdly*, one might postulate a necessary relationship between antibody specificity and L chain type. From Table IV, however, it is evident that among autoantibodies of a given L chain type there was no consistent pattern of reactivity with the genetically "deleted" cells. For example, the type II autoantibodies of De., Ad., and Sa. each clearly differed in these tests. *Finally*, a monotypic autoantibody population might be the product of a clone of cells which had escaped homeostatic controls (37). It is noted that the patients with monotypic autoantibodies included all 4 cases of chronic lymphocytic leukemia and the case of lymphosarcoma studied. L chain homogeneity in autoantibodies, however, may not necessarily imply their formation by pathologic mechanisms. The apparent L chain homogeneity of the anti-Rh_o antibodies from normal donor Gu. emphasizes the need to know the full spectrum of *normal* antibody responses in man before the L chain homogeneity of these autoantibodies can be placed in proper perspective.[10]

Because of the obvious limitations on interpreting homogeneity in such a simple two category system as the L chain types provide, other criteria of structural homogeneity, particularly other antigenic marker systems, are being applied to erythrocyte autoantibodies.

Table II also shows examples of autoantibody populations containing both type I and type II γ-globulins. The reasons for clearly bitypic responses in some AHD patients, as opposed to the monotypic responses in others, are not apparent. There is no obvious correlation with clinical diagnosis (Table II). Monotypic patterns were encountered both in idiopathic AHD and in cases associated with malignancy. The cases with clearly bitypic patterns are currently considered idiopathic. As a group, the patients with bitypic responses

[10] Donor Gu. is a 36-year-old female with rheumatic heart disease, but with no apparent hematologic disease, who had been sensitized by at least 3 Rh-incompatible pregnancies.

have had recognized erythrocyte autosensitization for a longer time (Table II). The groups are small, however, and determination of the date of onset of autosensitization may be difficult in retrospect. Any contribution of bitypic isoantibodies to the L chain typing of these autoantibodies is unlikely because the long intervals between blood transfusions and subsequent bleedings to obtain the eluates virtually eliminated any possibility of isosensitized donor RBC in these bleedings.

A possible explanation for the development of more heterogeneous (bitypic) autoantibody populations is the inclusion of 2 (or more) serologic specificities, with antibodies of each specificity carrying a different type of L chain. To investigate this point, the studies with genetically "deleted" RBC were initiated. The results to date confirm, first of all, the findings of Weiner and Vos (33) that such autoantibodies, which appear to be "pan-antibodies" on testing with ordinary RBC panels, may indeed show apparent specificity when tested with RBC deficient in the serologic expression of Rh antigens. No difference in specificities was found with these cells, however, between the clearly bitypic eluate of Po. and the monotypic type II eluate of Ad. (Table IV). Both reacted with all the test cells and were fully absorbed by the fully deleted (---/---) cells. In the Weiner-Vos scheme, therefore, these eluates might both be judged to have the same single specificity. The possibility, however, that reactivity with ---/--- cells could represent multiple specificities is recognized. One can only conclude that differences in specificity between these two antibody populations of different L chain content cannot be demonstrated in the test systems presently available.

Finally, one cannot exclude the possibility that a single cell line might produce autoantibodies of both L chain types. Of possible relevance, but perhaps too early to evaluate, are (*a*) the rare cases of myeloma associated with BJ and serum myeloma proteins of different L chain types (7, 38) and (*b*) the 1 to 3 per cent of normal lymphoid cells which might have contained both types of γ-globulins in the experiments of Bernier and Cebra (11).

There is no apparent reason to suspect that the elution and concentration procedures employed in this study prejudiced the L chain typing of the antibodies. All 3 possible L chain patterns were found in our AHD cases, and there was complete agreement in L chain typing of Rh antibodies derived directly from native serum and from eluates. Autoantibody eluates made by the 2 elution methods gave comparable results (patient De.).

"Incomplete" anti-Rh_o isoantibodies from 6 individual donors without known hematologic disease were chosen for parallel studies as normal antibody controls, for several reasons. First, like the autoantibodies under study, the Rh antibodies are 7S γ-globulins (see Materials and Methods) with maximum serologic activity at 37°C and with similar elution properties. Secondly, there is now mounting evidence implicating components of the Rh complex as de-

terminants for many RBC autoantibodies of the 7S class (33). Finally, from earlier observations (12), the known bitypic nature of Rh antibodies provided a valuable positive control.

Electrophoretic Dispersion.—The immunoelectrophoretic data accumulated to the present (Fig. 2) indicate that relative electrophoretic homogeneity may or may not be associated with L chain homogeneity in a given autoantibody population. Repeated observations of Ouchterlony reactions involving all the eluates shown in Fig. 2 did not suggest that differences in γ-globulin concentration were sufficient to account for the observed differences in electrophoretic dispersion. The autoantibodies of Fr., Re., and Po. and the anti-Rh_o antibodies of Bl. (Fig. 2) showed rather rapid electrophoretic mobility. The slow mobility of 7S γ-globulin in the eluate from patient Ad. suggests, however, that the elution procedure was *not* responsible for selecting populations of molecules with faster mobilities and thereby creating spurious electrophoretic homogeneity.

It is conceivable that the "monotypic" but electrophoretically polydisperse autoantibodies originate from two or more cell lines producing, perhaps by chance, L chains of the same type. It should be noted that certain antibodies made by *normal* individuals to selected antigens, such as dextran, may show some restriction of electrophoretic dispersion as compared to the whole serum γ-globulin population (39; see also reference 40).

Erythrocyte autoantibodies may thus possess varying degrees of structural similarity to "paraproteins." This relative homogeneity of structure appears to be more frequently encountered or more marked in populations of erythrocyte autoantibodies than among the anti-Rh_o and other "normal" antibody populations so far examined. Elucidation of the possible relevance of these findings to the pathogenesis of AHD will require further study.

SUMMARY

The 7S γ-globulins causing erythrocyte autosensitization in 20 patients were isolated by elution and examined for homogeneity or heterogeneity of their L chain types and electrophoretic dispersion. The isolated erythrocyte autoantibodies from 12 patients contained only 1 detectable L chain type. Two of these "monotypic" populations showed appreciable restriction of electrophoretic dispersion, while 2 others more nearly resembled the electrophoretic heterogeneity of normal γ-globulins. The autoantibodies from the other 8 patients exhibited L chains of both types. The single "bitypic" population so tested was relatively polydisperse electrophoretically. As a comparison, anti-Rh_o isoantibodies from 5 of 6 donors without known hematologic disease showed bitypic reactions, and 2 of these isoantibody populations were relatively polydisperse electrophoretically. One Rh isoantibody is described which contained only 1 demonstrable L chain type.

The structural similarities to "paraproteins" observed in a significant proportion of these erythrocyte autoantibodies raise the possibility of their origin from a restricted population of antibody forming cells, and may have implications concerning the pathogenesis of erythrocyte autosensitization.

The authors wish to express their indebtedness to Dr. John H. Vaughan for his sustaining encouragement and invaluable advice throughout the course of this study. Dr. Scott N. Swisher also made many valuable contributions to the development of the work.

The able technical assistance of Barbara Moyer and Janet Stone is gratefully acknowledged. Norma Trabold, William Marchetti, and Frank Buettner also assisted with certain phases of the work.

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