Nagoya J. Med. Sci. 47. 5 ~ 16, 1984

QUANTITATION OF RED-CELL-BOUND IgG IN NORMAL AND PATHOLOGIC STATES BY AN ENZYME IMMUNOASSAY (EIA) TECHNIQUE

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*This study was supported in part by a Grant-in-Aid
for Incurable Diseases "Idiopathic Hematopoietic Disorders"
from the Ministry of Health and Welfare, Japan.

ABSTRACT

A simple EIA technique for the quantitation of red-cell-bound immunoglobulin (IgG) was devised and applied to normal and pathologic states, including autoimmune hemolytic anemia (AIHA) and other disorders. The mean red-cell-bound IgG value for normal subjects was 73.2 ± 25.8 ng/10^{10} red cells (mean ± ISD). Fifteen patients with AIHA had an increased level of red-cell-bound IgG ranging from 109 to 10,000 ng/10^{10} red cells. The concentration of red-cell-bound IgG in AIHA patients was well correlated with the percentage of reticulocytes in the peripheral blood and showed a rapid fall after the initiation of steroid therapy. Red-cell-bound IgG levels in Fisher-Evans syndrome, idiopathic thrombocytopenic purpura (ITP), systemic lupus erythematosus (SLE), rheumatoid arthritis (RA) and IgG myeloma measured by this technique are also presented. A significant correlation was found between red-cell-bound IgG levels and serum IgG levels in normal subjects and in patients with these pathologic conditions, except in the cases of AIHA and Fisher-Evans syndrome. The quantitation of red-cell-bound IgG by this EIA technique is of great value for clarifying the pathophysiologic significance of red-cell-bound IgG in health and disease.

Keywords: Enzyme Immunoassay, Red-Cell-Bound IgG, AIHA.

INTRODUCTION

Although the quantitation of autoantibodies on red blood cells has been thought to be essential for clarification of the precise mechanism of *in vivo* hemolysis in AIHA, no reliable quantitation technique for red-cell-bound immunoglobulins was available until recent years. Constantoulakis *et al*[1] first attempted to estimate the amount of antibody on the red cells of AIHA patients using the radiolabelled antiglobulin test. Gilliland *et al*[2] and Rosse[3] assayed the amount of antibody on red cells by using the complement fixation technique as an indirect method. Various direct methods based on different principles have recently become available. Among these are included a PVP-potentiated Coombs test using an autoanalyser,[4] a method using continuous flowspacecytofluorometry,[5] a method employing radioimmunoassay of immunoglobulin[6] or complement[7] and a method employing EIA technique.[8-10] Previously, Kato *et al*[11,12] developed a highly sensitive sandwich enzymoimmunoassay of macromolecular

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Received for Publication January 30, 1984

antigens using Fab' fraction of rabbit antibody coupled to Beta-D-galactosidase together with $F(ab')_2$ fraction of rabbit antibody loaded on silicone pieces. Recently, we have succeeded in applying this sandwich EIA for the quantitation of the amount of IgG on the red cells. Here we report the technical details of the method and the results in the quantitation of IgG levels on the red cells in normal subjects and in patients with AIHA or other disease.

METHODS

Patients

Thirty-eight normal subjects and 54 patients were studied. The 54 patients studied were diagnosed as follows: warm-type AIHA with positive or negative Coombs test (15 patients); SLE with negative Coombs test (12 patients); Fisher-Evans syndrome (3 patients); IgG myeloma having serum IgG level of about 4g/dl (3 patients); RA (14 patients); and ITP (7 patients). Among the 15 patients with AIHA, 4 patients were studied before the initiation of therapy, 9 patients during the course of steroid therapy and two patients while off therapy and in remission. The AIHA patients were classified as idiopathic in 13 patients and secondary in 2 patients. Patient No. 1. shown in Table 1 was serially studied during the course of disease.

Preparation of Suspension of Red Cell Ghosts

Five ml of venous blood was collected in a plastic container using heparin as an anticoagulant. Red cells were separated by the Ficoll- Paque (Pharmacia Fine Chemicals, U.S.A.) density gradient method and the buffy coat was removed. Then the red cells were counted, washed six times in saline, and a 10% red cell suspension was prepared. After freezing and thawing the red cells, disrupted red cell ghosts were centrifuged at 35,000g for 20 min at 4°C. The precipitate was then washed twice with saline under the same conditions. The fragments of membranes obtained were resuspended to give a concentration equivalent to 10^{10} red cells/3 ml in 0.01M sodium phosphate buffer, pH 7.0, containing 0.1M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin and 0.1% NaN_3 (buffer A). They were finally treated by ultrasonication at maximum intensity for 10 sec in an ice bath. This suspension was prepared within 8 hours after the collection of the blood and was stored at –20°C until use. Additional ultrasonication was performed before each measurement. The stored red cell membrane suspension has continued to allow reproducible red-cell-bound IgG measurement after storage of more than 3 months.

Principle of Enzyme Immunoassay

Suspensions of red cell ghosts or aliquots of standard human IgG were incubated with silicone pieces and subsequently measured fluorometrically by the addition of Beta-D-galactosidase labelled rabbit antihuman IgG and 4-methylumbelliferyl-beta-D-galactoside (4MUG) as a substrate.

Preparation of Antibody-Loaded Silicone Pieces and Antibody-Enzyme Complex

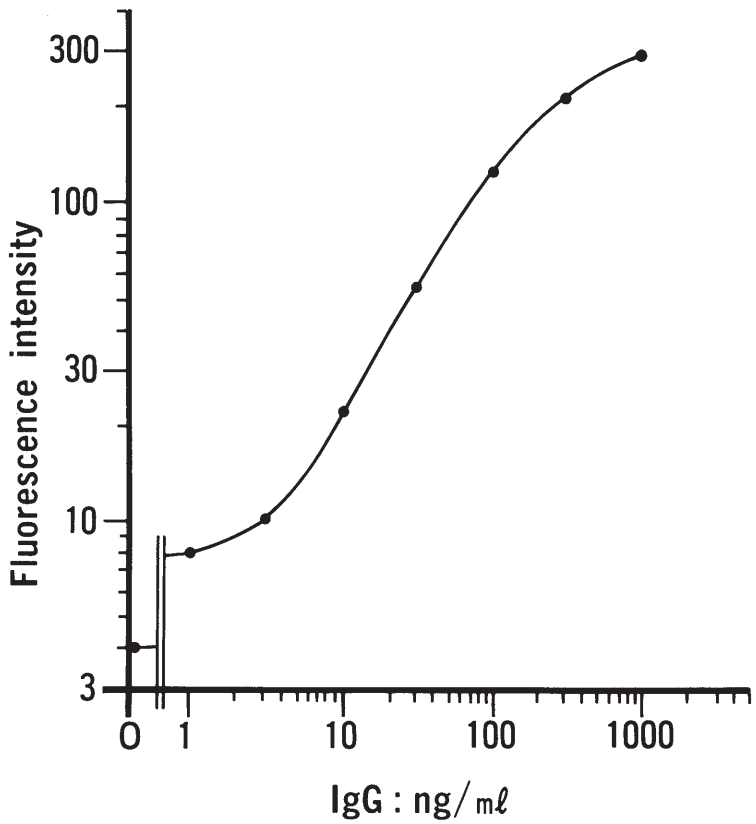
The $F(ab')_2$ and Fab' fragments of IgG were prepared from commercial gamma-chain-specific rabbit antihuman IgG (Cappel Laboratories, Inc., U.S.A.). The $F(ab')_2$ fragments were then loaded on silicone pieces. Fab' fragments prepared from the $F(ab')_2$ fragments were conjugated with beta-D-galactosidase from E.Coli using the method described by Kato *et al.*[11,12]

Assay Procedures of Red-Cell-Bound IgG

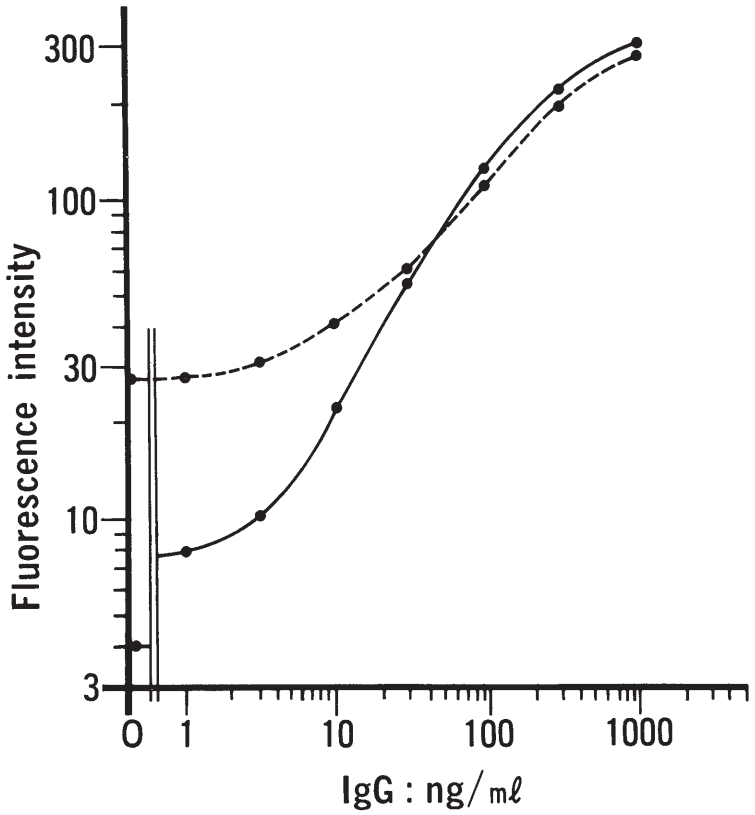
Each of the rabbit $F(ab')_2$ antihuman IgG-loaded silicone pieces was incubated in 0.4 ml of 0.01M sodium phosphate buffer, pH 8.0, containing 0.3M NaCl, 1 mM $MgCl_2$, 0.1% bovine serum albumin, 0.5% gelatin, 0.1% NaN_3 and 2 mM N-ethylmaleimide (buffer G) with 0.1 ml of suspension of red cell ghosts or standard IgG suspension. The mixture was incubated at 30°C for 3 hours with shaking. Each piece was washed twice in buffer A and incubated with antibody-enzyme complex in 0.15 ml of buffer A overnight and transferred to another test tube. After incubation of each piece with 50 ul of 10^{-4}M 4MUG for 10 min at 30°C with shaking, 2.5 ml of 0.1M glycin-NaOH buffer (pH 10.3) was added and the amount of 4-methylumbelliferone (4MU) formed was measured by a spectrofluorometer. Wave lengths used were 360 nm for excitation and 450 nm for emission analysis. All assays of the red-cell-bound IgG were performed in duplicate.

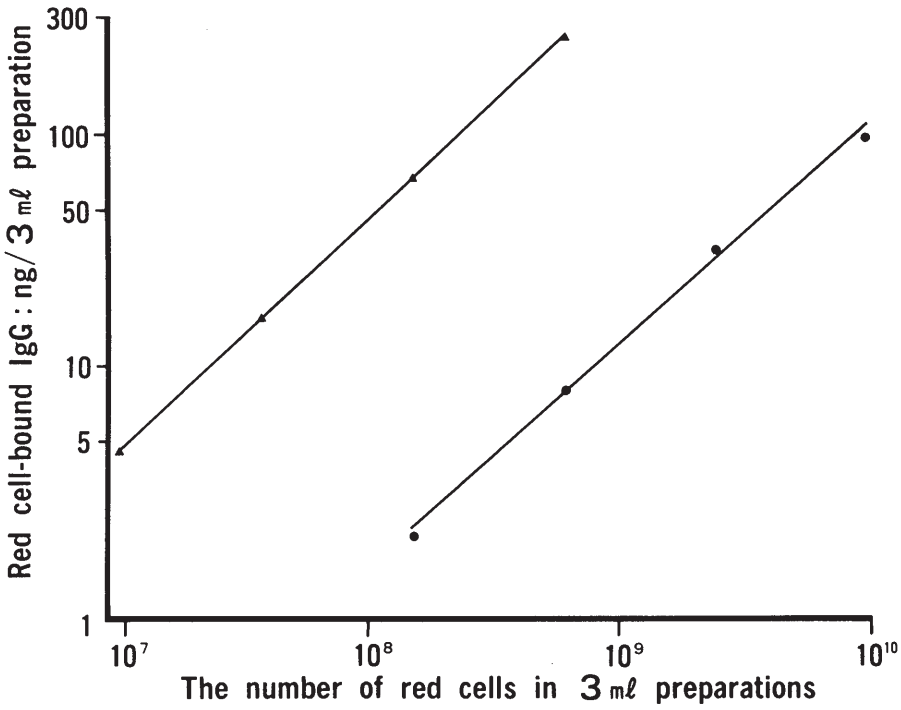
Precision of the Assay and Calculation of Red-Cell-Bound IgG

A sigmoidal standard curve was obtained between 1 and 300 ng/ml for soluble IgG (Fig 1). The values for red-cell-bound IgG were obtained from the standard calibration curve for soluble IgG (Fig 1). The concentration of red-cell-bound IgG was expressed in ng/10^{10} red cells. The



influence of the red cell membrane on the assay of soluble IgG was measured by adding known quantities of human IgG to a suspension of red cell ghosts. The reduction of soluble IgG values was found to be about 30% (Fig 2). When the value of red-cell-bound IgG was very high, an appropriate dilution of samples with buffer A was carried out. The value curve obtained from the diluted specimen was almost parallel to that obtained from the undiluted specimen. (Fig 3) The reproducibility of this assay was confirmed by duplicate testing of a normal (mean 44.4 ng, SD 2.6, cv 5.9%) and an abnormal sample (mean 10,000 ng, SD1,200, cv 12%).





RESULTS

The Quantity of Red-Cell-Bound IgG in Normal Subjects

The red cells from 38 normal donors were shown to have IgG values ranging from 35 to 120 ng/10^{10} red cells (mean 73.2 ng, SD 25.8) (Fig. 4).

The Quantities of Red-Cell-Bound IgG in Patients with AIHA and Other Diseases

AIHA (Fig. 4) (Table 1)

Table 1 shows the values of red-cell-bound IgG and the hematological data in 15 patients with AIHA. Prior to therapy, four patients with AIHA who had positive, direct and indirect Coombs test had cell-bound IgG values of 6,500, 7,000, 8,500 and 10, 000 ng/10^{10} red cells, respectively (mean 8,000.0 ng, SD 1,581.1). Serial measurements of red-cell-bound IgG, hemoglobin concentration and reticulocyte count were performed in one patient (No. 1) during the course of the disease (Fig. 5). A slow reduction of red-cell-bound IgG was observed during steroid therapy. In 9 AIHA patients undergoing treatment and who had only a positive direct Coombs test, red-cell-bound IgG values varied from 346 to 2,500 ng/10^{10} red cells (mean 1,349.0 ng, SD 813.4). In another two patients with AIHA, whose Coombs test became negative after steroid treatment, red-cell-bound IgG values were 109 and 204 ng/10^{10} red cells, respectively (mean 156.5 ng, SD 67.2).

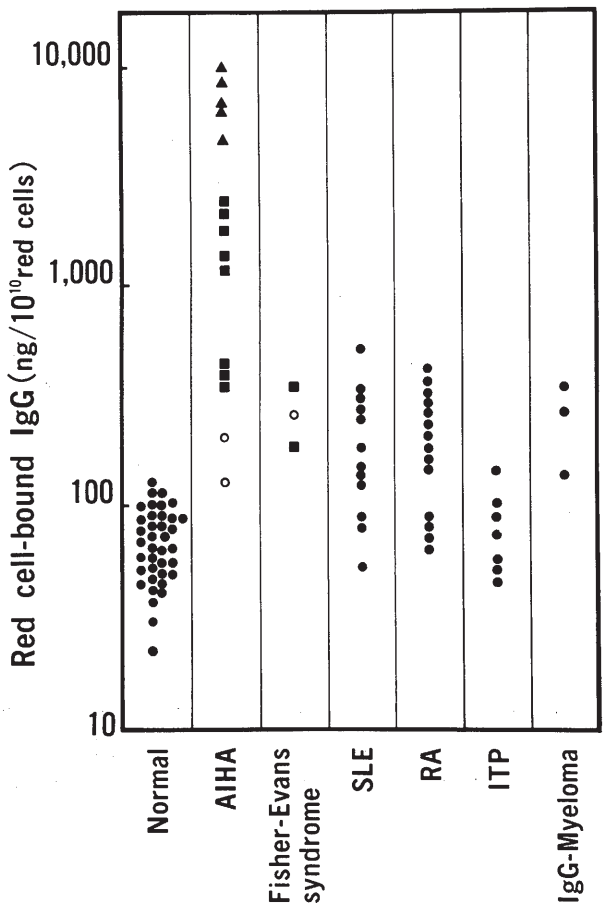
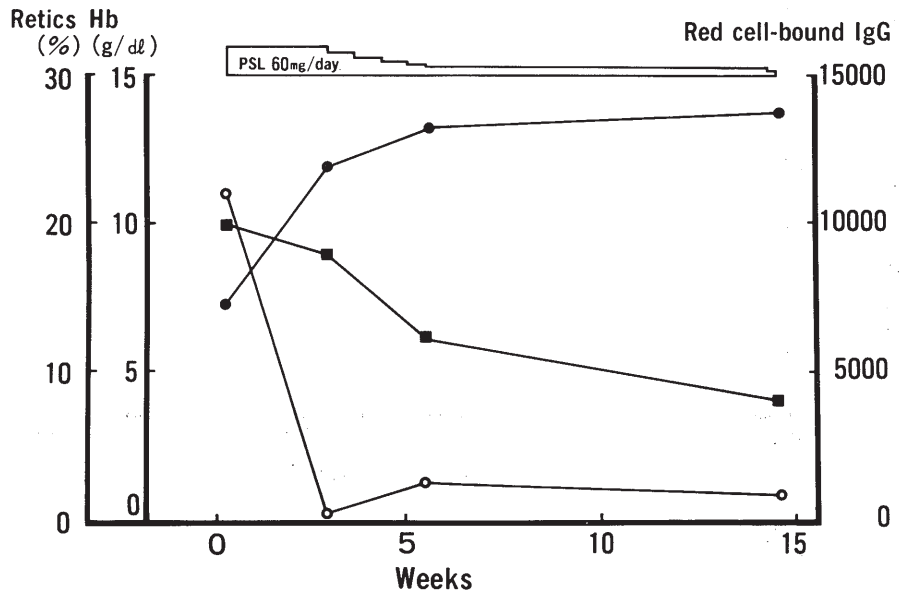


Table 1. Hematological and serological data of 15 patients with autoimmune hemolytic anemia

Case	Name	Age	Sex	Hb (g/dl)	Retics (%)	Red-cell-bound IgG (ng/10^{10} red cells)	Direct Coombs	Indirect Coombs
1	Y.M.	43	M	7.7	22.0	10000	(+)	(+)
2	S.W.	33	M	9.2	15.0	8500	(+)	(+)
3	K.O.	70	M	7.8	14.0	7000	(+)	(+)
4	T.H.	43	F	3.6	34.4	6500	(+)	(+)
5	Y.I.	42	M	11.2	6.2	2500	(+)	(−)
6	M.O.	78	M	15.7	6.6	2210	(+)	(−)
7	T.K.	42	F	12.1	0.6	1865	(+)	(−)
8	T.M.	70	M	6.7	15.3	1780	(+)	(+)
9*	N.B.	30	F	13.1	0.4	1340	(+)	(−)
10	A.M.	33	F	12.8	0.3	1280	(+)	(−)
11	M.T.	38	F	10.4	1.1	420	(+)	(+)
12	S.O.	55	M	7.8	8.8	400	(+)	(−)
13	K.T.	79	M	12.6	0.8	346	(+)	(−)
14*	S.A.	42	F	12.0	0.5	204	(−)	(−)
15	M.A.	54	F	10.0	0.4	109	(−)	(−)

*Secondary AIHA due to SLE



Fisher-Evans syndrome (Fig. 4)

Two patients with a positive direct Coombs test and a negative indirect Coombs test, had red-cell-bound IgG values of 188 and 360 ng/10^{10} red cells, respectively. Another patient whose Coombs test became negative after steroid treatment had a red-cell-bound IgG value of 260 ng/10^{10} red cells. The mean value in these groups was 269.3, SD 86.4.

SLE, RA, ITP, and Myeloma (Fig. 4)

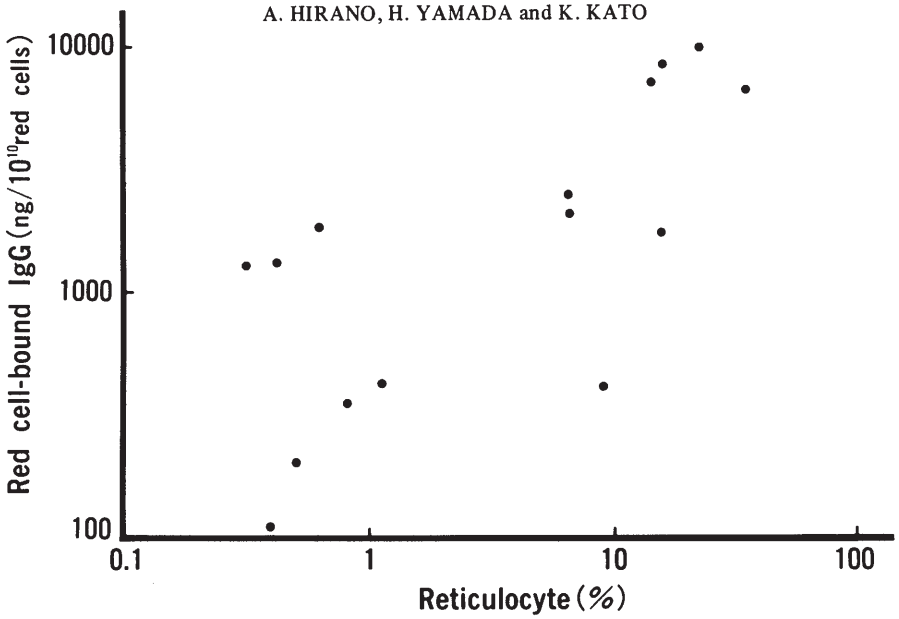
Twelve SLE patients with negative Coombs test had red-cell-bound IgG values ranging from 53 to 534 ng/19^{10} red cells (mean 214.8 ng, SD 137.8). Red-cell-bound IgG values in 14 patients with RA ranged from 68 to 414 ng/10^{10} red cells (mean 209.3 ng, SD 113.9). Seven patients with ITP had red-cell-bound IgG values ranging from 47 to 136 ng/10^{10} red cells (mean 81.2 ng, SD 32.0). Three IgG myeloma patients with serum IgG levels of about 4 g/dl had cell-bound IgG values ranging from 136 to 365 ng/10^{10} red cells (mean 223.0 ng, SD 124.0).

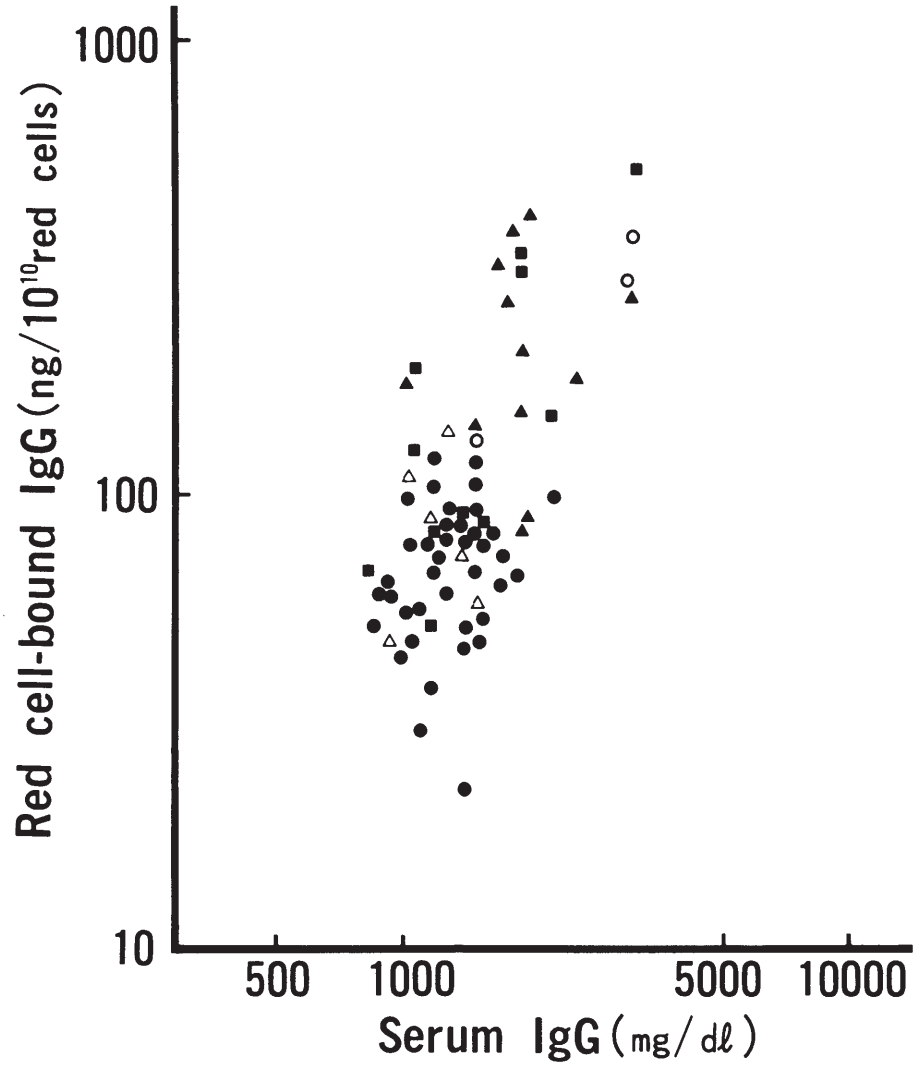
Correlation between Red-Cell-Bound IgG Value and Reticulocyte Percentage in Peripheral Blood in Patients with AIHA (Fig. 6)

A significant correlation was found between red-cell-bound IgG values and reticulocyte percentages of blood in 15 patients with AIHA ($r = + 0.770$, $p < 0{,}002$).

Correlation between Red-Cell-Bound IgG Value and Serum IgG Level (Fig. 7)

In normals and patients who had SLE, RA, ITP and IgG myeloma, a significant correlation was found between all their red-cell-bound IgG values and serum IgG levels ($r = + 0.720$, $p < 0.005$).


Red cell-bound IgG (ng/10^10 red cells)
10000
1000
100
0.1
1
10
100
Reticulocyte (%)


Red cell-bound IgG (ng/10^10 red cells)
1000
100
10
500
1000
5000
10000
Serum IgG (mg/dl)

DISCUSSION

We were able to detect as little as one ng of immunoglobulin on human red cell membranes by employing a technique based on the principle described by Kato *et al.*[11,12] The simplicity and reproducibility of this technique proved it to be an accurate and practical assay system for red-cell-bound immunoglobulin in the clinical laboratory. With this technique sonicated red cell membrane preparations can be used instead of red cell eluates. Although several techniques have been described for antibody elution from red cells, different methods are now employed for IgG and IgM antibodies on red cells. We prefer red cell membrane preparation to red cell eluate due to its simplicity and superiority in the IgM antibody assay. We can obtain reproducible results from sonicated red cell membranes prepared from a small volume of blood, (usually 5 ml) which can be stored stably at $-20^{\circ}C$ for many months. Batches of beta-D-galactosidase-antibody complex are also stable, and the same batch, once prepared, can be used over a period of many months. Values for cell-bound IgG in normals here coincide with values obtained by previous researchers using other methods.

We detected 19–39 molecules of IgG per red cell in normal subjects. The number of IgG molecules per normal red cell was 24–34 by Gilliland *et al*,[2] less than 35 by Tsunematsu,[6] less than 50 by Rosse,[13] less than 25 by Wilkinson *et al*[14] and less than 54 by Bodensteiner *et al*,[10] respectively. It is thus confirmed that a small but definite amount of tightly bound IgG is present on normal red cells. However, the biological significance of this red-cel-bound IgG remains to be elucidated, although some evidence has suggested its possible role in the mechanism of removal of senescent red cells[15] and in the IgG-binding function of the normal red cell plasma membranes.[16]

The present study demonstrated that patients with AIHA who had a positive direct Coombs test had a markedly increased level of red-cell-bound IgG in the range of 346–10,000 ng/10^{10} red cells. The levels of red-cell-bound IgG in most patients with AIHA, at diagnosis or in relapse, are high enough to be readily distinguishable from those in SLE and RA. We also demonstrated that there is a significant correlation between the amount of red-cell-bound IgG and the reticulocyte percentage in peripheral blood of AIHA patients. Constantoulakis *et al*[1] reported high and widely varying concentrations of antibody on the red cells of patients with Coombs positive AIHA using an anti-IgG labelled with ^{131}I, but there was no correlation between the amount of antibody on red cells and red cell survival time. However, Rosse[3] reported a clear relationship between the amount of red-cell-bound antibody and the rate of hemolysis in direct Coombs-positive AIHA. He further demonstrated that the alterations in the concentrations of red-cell-bound antibody and serum autoantibody were induced either by the administration of prednisolone or by the removal of the spleen. Gilliland *et al*[17] detected abnormal quantities of IgG ranging from 70 to 434 molecules on red cells from patients with Coombs-negative AIHA, most of whom achieved clinical remission after steroid therapy. In our study, a rapid or slow reduction in red-cell-bound IgG was observed during steroid therapy in three patients with AIHA. However, relatively high levels of red-cell-bound IgG persisted even after normalization of hemoglobin concentraiton. These facts may indicate that the administration of steroids reduces the quantity, though not to normal levels, of red-cell-bound IgG in patients with AIHA, along with normalizaiton of hemoglobin levels. From these findings in AIHA it has been shown that the quantity of IgG on red cells is one of the major factors in the rate of hemolysis *in vivo*, although it may occasionally be dependent on the characteristics of the individual autoantibodies. Furthermore, it has been shown that, in AIHA patients, more than 138 IgG molecules per red cell are required to make the Coombs test distinctly positive. This finding is in accordance with the fact reported by Dupuy *et al*[18] that as few as 250–500 molecules of IgG per cell are sufficient

to give a positive Coombs test. Red-cell-bound IgG can be used as a monitoring parameter during the treatment of individual patients with AIHA.

On the other hand, increased levels of red-cell-bound IgG were also detected in some patients with SLE, RA and IgG myeloma in this study. Half of the patients with SLE and RA had levels high enough to give a positive direct Coombs test, although none had evidence of hemolytic anemia. Also, two patients with IgG myeloma in this study had markedly increased levels of red-cell-bound IgG without evidence of hemolytic anemia, although Bodensteiner *et al*[10] recently reported normal red-cell-bound IgG levels in patients with IgG myeloma. Gilliland *et al*[2] demonstrated increased levels of red-cell-bound IgG in SLE and RA. In this study we found a significant correlation between red-cell-bound IgG and serum IgG level in both normals and patients with SLE, RA, ITP and IgG myeloma as a whole, although none had a correlation between red-cell-bound IgG value and reticulocyte percentage of blood. Thus, their red-cell-bound IgG does not always represent autoantibody but may represent isoantibody or non-specifically absorbed IgG in non-immune or immune processes. In this sense, a newer, direct assay technique such as the enzyme immunoassay described here may prove to be a useful tool to analyze the pathophysiologic roles of red-cell-bound IgG in various conditions. This method seems to be especially useful in AIHA since the amount of autoantibody on red cells is one of the major indicators of hemolysis in warm-type AIHA. This technique can also be used for detecting antibodies which are free in the serum or antigens on red cells to which various types of antibodies bind.

ACKNOWLEDGEMENTS

The authors are grateful to Professor Itsuro Sobue, 1st Department of Internal Medicine, Nagoya University School of Medicine, for his encouragement throughout this study and for his critical review of the manuscript.

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Fig. 1 Standard curve of soluble human IgG measured by the Beta-D-galactosidase labelled EIA. Fluorescence intensity: 100 = 10^{-7} M 4MU.

Fig. 2 Influence of red cell membranes on the EIA. Dashed line indicates IgG values when known quantities of soluble IgG were added to a suspension of red cell ghosts from a normal subject. Solid line indicates the standard curve of soluble IgG. Fluorescene intensity: 100 = 10^{-7} M 4MU.

Fig. 3 The number of red cells in serially diluted 3 ml preparations plotted against red-cell-bound IgG values derived from the standard curve of soluble human IgG. Typical results: normal sample (right) and sample from an AIHA patient (left).

Fig. 4 The quantities of IgG on the red cells from patients with AIHA and other disorders.
●: Normals, SLE, RA, ITP and IgG Myeloma patients (all negative direct Coombs test)
▲: AIHA patients with positive direct Coombs test prior to treatment
■: AIHA and Fisher-Evans Syndrome patients with positive direct Coombs test after treatment
○: AIHA and Fisher-Evans Syndrome patients with negative direct Coombs test after treatment.

Fig. 5 Serial measurements of red-cell-bound IgG, Hb concentration and reticulocyte percentage in a patient with AIHA (case No. 1) following administration of prednisolone (PSL).
■: Red-cell-bound
IgG; ●: Hb; ○: Retics.

Fig. 6 Correlation between red-cell-bound IgG levels and reticulocyte percentages in all patients with AIHA ($r = +0.770$, $P < 0.002$).

Fig. 7 Correlation between red-cell-bound IgG levels and serum IgG levels in normals and in patients with SLE, RA, ITP and IgG Myeloma ($r = +0.724$, $P < 0.005$).
●, Normals; ■, SLE; ▲, RA; △, ITP; ○, IgG Myeloma.