The Concentration of IgG in the Serum Is a Major Determinant of Fc-Dependent Reticuloendothelial Function

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Defective Fc receptor-specific reticuloendothelial (RE) function has been reported in certain patients with a variety of immunologic and nonimmunologic diseases. The mechanism responsible for the impaired RE function is uncertain, but it could be caused by immune complexes that are present in many of these disorders. Alternatively, the impaired RE function could be a secondary effect of the high concentrations of monomeric IgG in the serum of these patients, since monomeric IgG can compete with complexed IgG for macrophage receptors in vitro. We studied the Fc-dependent RE function in 30 healthy control subjects and 27 patients using IgG-coated radiolabeled autologous red cells. There was a significant relationship between the concentration of IgG in the serum and the rate of clearance of antibody-sensitized cells ($r = 0.51$, $P < .01$). Patients with hypergammaglobulinemia had the slowest Fc-dependent clearance, whereas those with hypogammaglobulinemia had the most rapid clearance. Immune complexes (Raji or polyethylene glycol) could not be shown to contribute to Fc-dependent RE clearance above the effect of the IgG in the serum. The usually rapid clearance in a patient with hypogammaglobulinemia could be returned to normal by raising the concentration of IgG in the serum. This study supports the concept that serum (monomeric) IgG competes with immune complexed IgG for macrophage Fc receptors in vivo. The competition for Fc receptors determines the level of competence of Fc-dependent RE function. Based on the results of this study, one can predict that a number of disorders characterized by hypergammaglobulinemia also will have impaired Fc-dependent RE function.

The reticuloendothelial (RE) system removes antibody-sensitized cells and particulate matter from the circulation. It plays a major role in defense against infection and participates in the clearance of autologous cells in certain autoimmune diseases. Clearance occurs through immune and nonimmune mechanisms. Antibody- or complement-sensitized cells are cleared via the respective Fc or C3b receptors on macrophages and monocytes of the RE system. The rate of clearance of antibody-sensitized cells varies with the amount and type of antibody and complement on the cell as well as with the functional integrity of the RE system. The amount of antibody and complement on the cell can be measured readily in vitro; however, techniques for measuring the Fc-specific clearance of the RE system in vivo are difficult and have only recently become available. The Fc-dependent function of the RE system can be estimated by measuring the rate of clearance of radiolabeled IgG-sensitized autologous red cells. Using this technique, investigators have demonstrated impaired RE function in a number of diseases, and it is possible that the impaired RE function contributes to the pathogenesis of certain immune complex-mediated disorders. For example, it has been postulated that defective Fc receptor function is an early event in certain autoimmune diseases. Consequently, immune complexes that otherwise would have been removed by the RE system circulate and deposit in the tissues. Alternatively, the concentration of immune complexes in certain disorders could be high enough to overload the Fc-dependent phagocytic capacity of the RE system. The circulating complexes could become tissue-bound, leading to tissue damage. Current experimental data neither confirm nor refute either hypothesis, and as yet a “common denominator” accounting for the impaired RE function has not been identified. It is also possible that the monomeric IgG in the circulation could compete with immune complexes for the Fc receptors of monocytes and macrophages. Although never shown in vivo, inhibition of the binding of aggregated IgG (immune complexes) by monomeric IgG has been demonstrated in vivo. Competition by “nonimmune” monomeric serum IgG for Fc receptors would offer an alternative explanation for the impaired Fc-dependent RE function in those autoimmune disorders characterized by polyclonal hypergammaglobulinemia. In this article, we describe a prospective study investigating the contribution of a number of serological and genetic factors to the Fc-dependent function of the RE system. The results indicate that the concentration of IgG in the serum is a major determinant of Fc-specific clearance. Patients with hypergammaglobulinemia had the slowest Fc-specific immune clearance, whereas patients with hypogammaglobulinemia had the fastest clearance. Treatment of a hypogammaglobulinemic patient with gammaglobulin returned the Fc-dependent clearance to normal levels.

MATERIALS AND METHODS

Patients and Controls

All studies were performed after informed consent was obtained. The study was approved following review by the University Ethics Review Committee and a Nuclear Physics Review Committee.

Healthy controls. The clearance of IgG-sensitized autologous red cells was studied in 30 healthy Rh-positive subjects. The clearance of heat-damaged autologous red cells was studied in eight Rh-negative subjects. No control subject was anemic, and all subjects had negative red cell direct antiglobulin tests and normal levels of platelet-associated IgG.

Patients. Thirty-six patients (27 Rh-positive and nine Rh-negative) with a variety of immunologic and nonimmunologic disorders were studied. Included in this group were 4 patients with hypogammaglobulinemia (common variable immune deficiency), 7 with chronic liver diseases, 7 with systemic lupus erythematosus, 17
with rheumatoid arthritis, and 1 with a chronic infection (abscess).
None of the patients had undergone splenectomy or none had thrombocytopenia or immune hemolytic anemia as evaluated by a positive direct antiglobulin test. All of the hypogammaglobulinemic patients were well adults and none had chronic infections.

In Vivo Studies

Determination of RE cell function. The function of the reticuloendothelial cell system was assessed by measuring the clearance of IgG-sensitized, $^{51}$Cr-labeled autologous red cells if the subject was Rh-positive. Rh-negative subjects were evaluated by determining the clearance of $^{51}$Cr-labeled heat-damaged red cells. All labeling procedures were performed under sterile conditions.

Whole blood from an Rh-positive subject was collected in ACD (6:1, vol/vol), and the hematocrit was determined on a subsample. The hematocrit was used to calculate the amount of whole blood required to give 2 mL of packed red cells. This amount of whole blood was centrifuged (1,550 g for ten minutes) and the plasma discarded. The erythrocytes were incubated (37 °C for ten minutes) with 25 μCi $^{51}$Cr at 37 °C, washed three times, and then incubated (37 °C for 15 minutes) with 2 mL of varying dilutions of anti-D (WinRho, Rh Institute, Winnipeg, Manitoba, Canada). In the initial series of experiments on the relationship of the degree of sensitization to the clearance time, varying dilutions of anti-D were used (from 1:50 to 1:200). Subsequently, all experiments done in the patients used 2 mL of a 1:100 dilution of anti-D for red cell sensitization. This amount consistently resulted in binding ranging from 1,500 to 4,000 molecules of IgG per red cell. The red cells were washed twice with 0.9% sodium chloride, the supernatant was discarded, and the cells were resuspended to 6 mL. Subsamples were taken for determination of the red cell count, the red cell specific activity, and the quantitation of IgG per red cell. Each subject received an average of 1.7 (±0.22) × 10¹¹ red cells (mean ± SD, n = 10). Following infusion of the red cells, a 3-mL whole blood sample was collected into EDTA at 5, 10, 15, 20, 30, and 60 minutes for measurement of red cell radioactivity. The whole blood radioactivity was plotted on semi-log paper against the time following infusion of the red cells with the 100% point being the whole blood radioactivity at five minutes. Both the area under the curve and the percentage of red cells remaining in the circulation at 60 minutes were used in the calculation of clearance rates.

Whole blood from an Rh-negative subject was handled in a similar fashion, except that the red cells were heat-damaged by incubation at 49.5 °C for 20 minutes before to labeling with $^{51}$Cr.

In Vitro Studies

Red cell-associated IgG. The amount of IgG on the red cells was determined using an immunoradiometric assay. This technique is a modification of the immunoradiometric assay for platelet-associated IgG. One hundred microliters of $^{125}$I-anti-IgG was incubated (37 °C for 60 minutes) with a series of dilutions of red cells washed four times and suspended in 0.15 mol/L of pH 7.4 phosphate-buffered saline (PBS). One hundred microliters of IgG beads (10⁶ beads) was added and incubated for a further 30 minutes at 37 °C. The IgG beads were prepared by covalently binding human IgG (Cohn Fraction II, Sigma Chemicals, St Louis) to agarose beads (Sephasorb HP-Ultraline, Pharmacia Fine Chemicals, Dorval, Quebec) using carbonyl-diimidaole. The red cells were lysed with 100 μL of 1% Triton X-100, the beads washed twice with 1.5% bovine albumin and the radioactivity was measured in a gamma counter. A standard curve was prepared using a similar protocol, except that increasing concentrations of human IgG standard were used (10 to 500 ng). The amount of IgG per red cell was calculated by dividing the concentration of IgG standard that caused 50% inhibition of binding of the $^{125}$I-anti-IgG to the IgG beads by the number of red cells that also caused 50% inhibition. The results were expressed as weight of IgG per 10⁶ red cells.

Determination of HLA and DR alloantigens. The HLA type of the controls and patients was determined using antisera provided by the National Centre of the Canadian Red Cross Blood Transfusion Service. The DR alloantigens were determined on 8 lymphocytes, using anti-DR-specific alloantisera provided by the National Reference Laboratory of the Canadian Red Cross Blood Transfusion Service.

Serologic tests. Immune complexes were measured using the Raji cell assay and the polyethylene glycol (PEG) assay. The Raji cell assay was performed using Raji cells harvested after 72-hour subculture. Neat and diluted serum samples were incubated at 4 °C for one hour. The cells were washed three times, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-human immunoglobulin. Positive cells were detected with a fluorescent microscope. Heat-aggregated human IgG served as positive control.

PEG assay was performed by mixing test serum samples with 5% PEG 6000. The solution was left for 60 minutes at 37 °C and then at 4 °C overnight. The samples were centrifuged, and the precipitate was washed with 2% PEG solution. The precipitated complexes were dissolved in saline, and their IgG content was measured nephelometrically using specific anti-IgG antisera. Except for certain myeloma sera, the method was independent of serum IgG levels over a wide range.

Antinuclear antibody was measured using standard techniques. Serum immunoglobulin levels (IgG, IgA, IgM) were measured nephelometrically.

Statistical analysis. In order to determine whether variables were significantly correlated with one another, Pearson's correlation coefficients were computed and t tests were done to assess statistical significance. In addition, a stepwise multiple linear regression was performed, using program BMDP2R (BMDP, Biomedical Computer Programs, 1979) to assess those factors important in predicting the red cell clearance at one hour. The rationale for this analysis is that although there may be several factors that alone are highly predictive of the outcome, a combination of factors may be better than any single factor alone. Alternatively, a combination of factors may not add to the prediction of the outcome. This situation can arise when the predictors themselves are correlated. The stepwise multiple regression identifies that factor most highly correlated with outcome, and assesses the statistical significance of that relationship. It then successively selects from the remaining factors the one that adds the most in explaining the outcome over and above those factors already in the prediction model. The proportion of variance in the outcome explained by the factors in the prediction equation is expressed as $r^2$. When there is only a single term in the model, the term is simply the square of the Pearson correlation coefficient between predictor and outcome. The statistical significance of each successively added term is assessed by applying an F test on the change in $r^2$ as a result of adding the new term to the prediction model.

RESULTS

Thirty-eight different healthy men and women control subjects were tested. The control subjects included 30 Rh-positive and eight Rh-negative subjects. The antinuclear antibody (ANA) assay and Raji cell assay for immune complexes was negative in all. One Rh-positive control patient had circulating immune complexes as assayed by PEG assay. This test was negative in all other controls. The serum IgG was within normal limits for all. Five of the
Rh-positive and two of the Rh-negative control subjects had the HLA-B8/DR3 haplotype.

The relationship between the amount of red cell sensitization and the clearance of the red cells for the 32 tests performed on the 30 Rh-positive control subjects sensitized with varying amounts of anti-D is shown in Fig 1. There was a significant correlation between these two parameters with the best fit being a linear relationship between clearance and the log of sensitization ($r = -0.709, P < .001$). Greater sensitization resulted in a more rapid clearance.

The Fc-dependent clearance of chromium-labeled, autologous red cells in patients were performed, using moderate sensitization (2 mL of a 1:100 dilution of anti-D). In every patient, this resulted in red cell sensitization of 1,500 to 4,000 molecules of IgG per red cell. A moderate level of sensitization was chosen for the patient studies, since low levels of sensitization resulted in a slow rate of clearance of the sensitized red cells, whereas high levels of sensitization resulted in a very rapid rate of clearance (Fig 2). We postulated that clearance studies performed at either extreme of sensitization technically could be more difficult to perform and interpret. The 17 healthy Rh-positive control subjects whose red cell clearance studies were performed with similar levels of sensitization (1,500 to 4,000 molecules per cell) served as controls for this group. The mean percentage of sensitized red cells remaining in the circulation of these healthy controls at 60 minutes following infusion was $52.6 \pm 4.9$ (mean $\pm$ SE) (Fig 3).

Thirty-six Rh-negative and Rh-positive patients were studied. Ten of the Rh-positive patients and five Rh-negative patients had circulating immune complexes by one or both assays (Fig 2). In seven patients, immune complex assays were not performed. Nine patients were ANA-positive (seven Rh-positive and two Rh-negative). Four Rh-positive and three Rh-negative control subjects had the HLA-B8/DR3 alloantigens.

The analysis of clearance of the IgG-sensitized cells was evaluated in two ways: by determination of the area under the curve (AUC); and by measuring the percentage of sensitized cells remaining in the circulation at 60 minutes after infusion. The correlation between these two parameters was $r = .96$. Neither estimate was judged superior to the other, and to simplify comparison of these results with previous studies, all subsequent results were expressed as the

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**Fig 1.** The relationship between the clearance of $^{51}$Cr-labeled IgG-sensitized autologous red cells (ordinate) and the amount of IgG on these cells (abscissa) for 32 tests performed on 30 different healthy control subjects ($r = -0.82, P < .001$). A better relationship was observed when the clearance was related to the log of the sensitization, $r = -0.709$. The five controls with the HLA-B8/DR3 haplotype are shown as [□]. One HLA-B8/DR3 control was tested twice.

**Fig 2.** The clearance of IgG-sensitized red cells (ordinate) for control subjects ($n = 17$) [□] and patients ($n = 27$) [○] in relationship to the serum concentration of IgG (g/L). [□] indicates the patient or control subject has the HLA-B8/DR3 haplotype; [□] indicates a positive polyethylene glycol assay for immune complexes; [□] indicates a positive Raji cell assay for immune complexes; [□] indicates a positive assay for antinuclear antibodies.
percentage of labeled cells remaining in the circulation at 60 minutes postinjection.

Stepwise linear regression was used to examine the relationship between the percentage of red cells remaining in the circulation at 60 minutes in the 27 different Rh-positive patients and: (1) the amount of IgG sensitizing the red cells; (2) the serum concentration of IgG; (3) the presence of immune complexes (PEG and Raji assays); and (4) the results of the ANA test. The only significant relationship was between the concentration of IgG in the serum and the percentage of recovery at 60 minutes ($r = .51, P < .01$). The presence of immune complexes (Raji or PEG) or a positive ANA could not be shown to provide an additional contribution. Although there was a significant correlation between the clearance at 60 minutes and the presence of circulating immune complexes ($r^2 = .41$), this could be due to the association between the presence of immune complexes and the serum concentration of IgG. To determine statistically whether one of the variables could account for the other’s effect on clearance, each was partialed out of the analysis and the data were reanalyzed. Partialing out the effect of serum IgG had a net result of cancelling out the effect of positive immune complexes on clearance. In contrast, even after the effect of immune complexes was partial out of the analysis, there remained a significant relationship between clearance and serum IgG concentration ($t_{19} = 1.85, P < .05$, one-tailed).

The greatest impairment of clearance of IgG-sensitized red cells occurred in those patients with the highest concentration of IgG in their serum irrespective of the presence of immune complexes or a positive assay for ANAs (Fig 2). In contrast, the most rapid clearance occurred in the patients with hypogammaglobulinemia. The clearance curve of one patient with hypogammaglobulinemia before and following the administration of gamma globulin is shown in Fig 3. Treatment elevated the concentration of IgG in the serum from 2.2 to 4.0 g/L, and the previously shortened clearance rate increased to within the normal gap.

The clearance of chromium-labeled, heat-damaged red cells was determined in Rh-negative healthy control subjects and patients (Fig 4). Neither the level of serum IgG, the presence of immune complexes, the HLA-B8/DR3 haplotype, or a positive ANA test influenced clearance.

The influence of the HLA-B8/DR3 haplotype on the function of the RE cell system was not mathematically evaluated because of the small number of patients with these alloantigens; however, at the same concentration of serum IgG, clearance tended to be more impaired in control subjects and patients with the HLA-B/DR3 alloantigens as compared with persons who did not have these alloantigens.

**DISCUSSION**

The RE cell system is a major participant in host defense and autoimmune disease. The factors controlling its function are not well understood, and techniques for evaluating RE function in vivo have only recently become available. Fc-dependent RE function can be evaluated by measuring the clearance of radiolabeled IgG-sensitized red cells. This technique has been validated both in animals and humans, with clearance depending upon the interaction of the Fc portion of the IgG molecules and Fc receptors on phagocytic cells. Investigators have used this method to demonstrate abnormal RE function in patients with a variety of autoimmune diseases, including systemic lupus erythematosus, rheumatoid arthritis, and certain chronic hepatic disorders. The impaired RE function in patients with systemic lupus erythematosus tends to be the greatest in those patients with the greatest disease activity. Many of these patients also have circulating immune complexes. It is possible that the impaired Fc-dependent RE function results in the reduced clearance of immune complexes, which in turn participate in the autoimmune disorder. Alternatively, the presence of the immune complexes and the impairment of RE function could be related to other uncharacterized variables. The current study supports the latter hypothesis and shows that the concentration of serum IgG is the most important factor affecting Fc-dependent RE function. This conclusion was based upon the study of Fc-dependent RE function in control subjects and patients. The patients with the highest concentration of serum IgG had the slowest RE clearance, whereas those patients with hypogammaglobulinemia had the most rapid clearance (Figs 1 and 3). The demonstration that the unusually rapid clearance in a hypogammaglobulinemic patient returned to normal as the concentration of IgG in the serum was raised is also consistent with this hypothesis (Fig 3). The work of Fehr and coworkers also supports our conclusions. These investigators raised the serum concentration of IgG to supranormal levels in a group of patients with immune thrombocytopenia and these patients had a transient remission in their disease, presumably due to RE blockade. Thus, the studies described in this report relating RE function in patients with naturally occurring serum gamma globulin concentrations that ranged from abnormally low to abnormally high and Fehr’s observa-
tion in patients with therapeutically elevated concentrations of IgG support the concept that serum IgG is the major determinant of Fc-dependent RE function. To challenge this hypothesis more directly, we analyzed our observations by using stepwise multiple linear regression analysis. This analysis tests whether a single factor, or a combination of factors can predict an outcome—in this case, Fc-dependent RE function. This type of analysis is useful when one or more factors are associated with an outcome, but themselves may be correlated. We found that the concentration of IgG in the serum was a sufficient explanation for RE clearance, and the addition of other variables such as positive immune complexes or a positive antinuclear antibody test did not add to the prediction model. To challenge the conclusion further, the analysis was repeated after any impact of immune complexes on RE clearance was mathematically partialed out. A significant relationship between serum IgG and RE function still remained.

Our observations could, in part, explain the results of previous investigators. For example, one group of investigators reported rapid improvement of Fc-specific clearance following plasma exchange in patients with systemic lupus erythematosus. Plasma exchange with replacement using either albumin or plasma from healthy donors would likely produce a sudden and dramatic reduction in the concentration of IgG in the serum of the patients.

Our observations should not be interpreted as indicating that immune complexes do not affect Fc-dependent RE function. The binding affinity of immune complexes to monocytes and macrophages is higher than the affinity of monomeric IgG for these RE cells. However, because the interaction of immune complexes or monomeric IgG with Fc receptors obeys the Law of Mass Action, the relative concentration of each is important. Consequently, we believe the very high concentration of monomeric IgG in serum (10⁻⁴ mol/L) overwhelms the effect of immune complexes. Consistent with this conclusion are the observations of Segal and...
co-workers. These investigators reported that, in vitro, the binding of immune complexes to macrophages could be inhibited by monomeric IgG.\textsuperscript{5} And yet IgG is not the only determinant of Fc-dependent RE function. For example, others have demonstrated that patients and controls with the HLA-B8/DR3 haplotype have impaired RE cell function.\textsuperscript{13} While not tested directly because of small numbers, our results (Figs 1 and 3) are consistent with this observation.

Although the concentration of IgG in the serum was shown to be a major factor controlling the rate of clearance of IgG-sensitized cells, a similar relationship could not be demonstrated for heat-damaged red cells. An observation suggesting that the clearance of abnormally shaped red cells is mediated by different mechanisms (ie, splenic sieving) and not by Fc-mediated clearance.

This study also provides indirect information concerning the way in which the monocyte/macrophage Fc receptor recognizes the Fc portion of the IgG molecule. To remove an antibody-sensitized particle (immune complex or antibody-bound cell), the RE cell must differentiate complexed IgG from the monomeric IgG in the plasma. Two theories have been proposed to explain this phenomenon. The allosteric theory suggests that the binding of an antibody to an antigen causes a change in the Fc portion of the immunoglobulin molecule producing a higher affinity for the Fc receptors. The aggregative theory proposes that the binding of IgG to an antigen does not result in a conformational change in the antibody. Rather, the antigen increases the relative concentration of IgG presented to the macrophage/macocyte Fc receptors. This latter hypothesis suggests that the monocyte/macrophage cannot differentiate between free or bound IgG (immune complex), and it is the relative concentration of IgG presented to it that determines binding. The current study indirectly favors the aggregative theory. Patients with hypogammaglobulinemia have lower serum concentrations of IgG and, consequently, there is less IgG to compete with the IgG-sensitized erythrocytes for RE Fc receptors. In contrast, in those patients with high concentrations of serum IgG, the increased monomeric serum IgG competes with the IgG-sensitized red cells for macrophage/macocyte Fc receptors.

We have previously described patients with elevated levels of platelet-associated IgG but normal platelet survival.\textsuperscript{8} All of these patients had hypergammaglobulinemia and impaired Fc-dependent RE function. One can develop a model to explain the results of our previous report and the current studies.

We believe that at least in some patients an equilibrium exists among IgG in the serum, IgG bound to circulating cells, and IgG bound to the Fc receptors of RE cells. A rise or fall in the plasma concentration of IgG results in a parallel rise or fall in the “baseline” cell-bound IgG and the IgG interacting with the Fc receptors of the RE cells, illustrated diagrammatically in Fig 5. Consequently, there is no net change in cell clearance. Increased clearance occurs when one “arm” of the equilibrium is disrupted, for example, when a disproportionate rise in cell-bound IgG occurs as in certain immune cytopenic disorders.

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