Protein – Quinone Interaction: In Vitro Induction of Indirect Antiglobulin Reactions With Methyldopa

By Arlan J. Gottlieb and Harold A. Wurzel

Methyldopa-treated gamma globulin can be demonstrated serologically on either the red cell surface or on latex beads by the indirect antiglobulin reaction. The development of a positive antiglobulin reaction was related to methyldopa concentration and the length and temperature of incubation of methyldopa with protein and could be partially inhibited by the addition of albumin to the incubation mixtures. After more prolonged incubation, antiglobulin positivity also developed with plasma-treated with methyldopa. 14C-methyldopa was covalently bound to gamma globulin. Aggregation of gamma globulin following treatment with methyldopa could be demonstrated by both sedimentation velocity and molecular weight determinations employing low-speed equilibrium centrifugation. Protein aggregation was a function of time, temperature, and methyldopa concentration. Detectability by the antiglobulin reaction, the darkening noted in solutions to which methyldopa or hydroquinone had been added, as well as the aggregation of protein was inhibited by a reducing agent which prevented formation of a quinone from the hydroquinone. Some of the immunologically atypical features of the sensitization of red cells by methyldopa or its structural analogues are explicable by the adherence, in vivo, of chemically modified, non-antibody gamma globulin which renders the red cell directly antiglobulin positive.

A POSITIVE ERYTHROCYTE antiglobulin reaction occurs in approximately 10%-20% of patients receiving α-methyldopa (methyldopa) (1-α-methyl 3,4 dihydroxyphenylalanine).1-5 The direct antiglobulin reaction usually becomes positive 3-6 mo after the initiation of therapy with standard doses of methyldopa and reverts slowly after its withdrawal.2 Surprisingly, overt hemolysis is rare, and an anamnestic response is not seen when methyldopa is readministered to previously antiglobulin positive individuals.4

Circulating antibodies may occasionally be demonstrated,3,5,6 and gamma globulin having apparent antibody specificity for the c, e, and D determinants of the Rh locus has been eluted from the sensitized red cell.5,5 In other studies, red cell eluates proved to be panagglutinins for the Rh locus but were unreactive with Rh null cells or cells from lower order primates that lack Rh antigens.7 As a result of these studies which fail to indicate antibody specificity directed toward methyldopa but which do indicate an Rh specificity for the gamma globulin eluates of sensitized red cells from methyl-
Dopa-treated individuals, a concept of drug-induced "autoimmunity" has been advanced to explain the development of positive antiglobulin reactions in patients receiving methyldopa. In other studies, methyldopa has been found to be broadly heterospecifically haptenic in its antigenicity in the rabbit. It has also been shown that positive direct antiglobulin reactions may be induced in vitro by the incubation of erythrocytes or blood with methyldopa or related catecholamines. These latter studies suggest that chemical, rather than immunologic, mechanisms may be responsible for the induction of antiglobulin positivity. The present study was undertaken to evaluate the possibility that reactions between gamma globulin and quinones can render gamma globulin serologically detectable on the red cell surface and to determine the nature of any structural change which might have occurred in the gamma globulin.

MATERIALS AND METHODS

Pure methyldopa and 14C-methyldopa (specific activity 11.9 mCi/mg) were obtained from Merck, Sharp & Dohme. Gamma globulin obtained commercially (Pentex) or prepared by fractionation of normal human serum with 18% sodium sulfate was further purified by chromatography on DEAE-cellulose. The product was pure IgG immunoelectrophoretically. The sedimentation coefficient (S20,w) was 6.5-6.8 S and the molecular weight was 158,000-168,000 (vide infra).

The remainder of the chemicals used were of reagent grade. Urea solutions were clarified and deionized as described by Clegg et al. An antibody prepared against the heavy chain of gamma globulin was employed as antiglobulin reagent. The antibody was absorbed with A and B substance and light chains of gamma globulin (kindly provided by Dr. Neva Abelson).

Induction of Positive Indirect Antiglobulin Reactions

Normal human serum or gamma globulin (10 mg/ml) in 0.01 M sodium phosphate and 0.14 M NaCl at pH 7.4 (phosphate-buffered saline) was incubated at 25°C with methyldopa (0.01 to 5.0 mg/ml). At intervals, 0.2 ml of solution was removed and incubated for 1 hr with either compatible or type O red cells at 37°C. The red cells were then washed three times and the standard antiglobulin reaction performed. Normal human serum or gamma globulin served as controls for these studies. In some experiments, stoichiometric equivalents of p-hydroquinone were substituted for methyldopa. In others, molar equivalents of sodium ascorbate were added to solutions containing methyldopa. The antiglobulin reaction were scored on a 1 to 2+ basis. A 1+ reaction denotes microagglutination, while a 2+ reaction indicates strong microagglutination.

Thoroughly washed latex beads (9 μ) were also employed as a carrier particle for the antiglobulin reaction. Either serum, or 1% gamma globulin solutions were treated with methyldopa as noted above. When serum was employed, 1 ml of the methyldopa-serum mixture was fractionated with 18% sodium sulfate. The precipitate was washed with 18% sodium sulfate, dissolved in borate buffer pH 8.5, mixed with the latex beads, and allowed to stand overnight at 4°C. Serial dilutions of methyldopa-treated gamma globulin were employed to obtain titers. After the overnight incubation, the beads were thoroughly washed and then tested for agglutination with antiglobulin reagent.

The effect of albumin concentration upon the development of positive antiglobulin reactions was studied by incubating 0.5 mg/ml of methyldopa with a series of 1% gamma globulin solutions containing increasing concentrations of purified bovine serum albumin. Alternatively, 0.5 mg/ml methyldopa and gamma globulin were incubated and varying concentrations of albumin added at the end of the incubation period. The ability to sensitize red cells was tested as described above.

Binding of Methyldopa to Gamma Globulin

Gel filtration studies. Gamma globulin (80 mg) was incubated with 1 mg 14C-methyldopa for 16 hr at 25°C in 3 ml, dialyzed, and applied to a column of Sephadex G-200 equilibrated against the same buffer. Radioactivity in the column effluent was determined by placing aliquots from each tube in scintillation fluid (BBOT in toluene) and counted in a liquid scintillation counter. Further details are given in the legend to Fig. 1.
Forty milligrams of $^{14}$C-methyldopa-treated gamma globulin was prepared in a similar manner and then extensively dialyzed against a solution of 8 M urea, 0.05 M phosphate, and 0.1 M NaCl at pH 7. Following dialysis the protein was applied to a 2.5 x 50-cm column of equilibrated Sephadex G-100.

**Dialysis studies.** Binding of methyldopa to gamma globulin was studied by dialysis to equilibrium in both 0.25 M sodium borate buffer pH 6.7 and in a solution of 8 M urea-0.25 M sodium borate at 6.7. One-tenth milligram of $^{14}$C-methyldopa was allowed to react to 80 mg gamma globulin at 25°C for 24 hr in 0.25 M borate in a volume of 2 ml. Aliquots were then dialyzed against either borate or the urea-borate buffer. Seventy-two hours of dialysis against three changes of 125 ml of borate or urea-borate buffer were employed. The experiment was terminated when the radioactivity in the dialysate was constant. The binding of $^{14}$C-methyldopa to protein was calculated from the radioactivity in both the dialysate and dialysand.

**Trichloroacetic acid precipitation studies.** When trichloroacetic acid (TCA) precipitation was employed to study $^{14}$C-methyldopa binding to gamma globulin, 0.1-ml aliquots were removed from a 25°C reaction mixture containing 10 mg/ml of gamma globulin and 0.5 mg/ml $^{14}$C-methyldopa and placed in 1 ml 10% TCA. The precipitate was washed twice with 10% TCA, dissolved in sodium hydroxide, placed in scintillation fluid, and radioactivity determined.

**Binding to Gamma Globulin Fragments**

To determine the binding of methyl dopa to the Fab and Fc fragments of gamma globulin, 10 mg of $^{14}$C-methyldopa were allowed to react with 200 mg of gamma globulin in 0.05 M phosphate-0.1 M NaCl, pH 7 for 24 hr at 25°C. The protein was dialyzed, freeze dried, and dissolved in 0.05 M sodium phosphate buffer containing 0.1 M NaCl, 0.01 M cysteine, and 0.002 M EDTA at pH 7, prior to digestion overnight at 37°C by 2 mg of papain. The Fab and Fc fragments were separated chromatographically on carboxymethyl cellulose. Optical density and radioactivity of the effluent fractions were determined. Each component was identified immunoelectrophoretically utilizing specific antisera (provided through the courtesy of Dr. Steven Douglas).

**Determination of Apparent Change in Protein Concentration by Sedimentation Velocity**

Equal volumes of methyldopa-treated and untreated gamma globulin solution containing 10 mg/ml of protein were placed in single sector cells of a Model E analytic ultracentrifuge. Sedimentation velocities were determined at 56,000 rpm and 20°C employing schlieren optics and a positive wedge window in one cell. A single sedimenting peak was obtained in each centrifuge cell. The area under each peak (6.5-6.85 S) was determined planimetrically from an enlarging traced of the photographic record. Studies involving variations in methyldopa concentration, time, and temperature of incubation were made. The effect of the simultaneous addition of ascorbate (0.1 mg/ml) and methyldopa (0.1 mg/ml) to gamma globulin was studied.

**Effect of Methyldopa Upon Molecular Weight of Protein**

The molecular weight of methyldopa-treated gamma globulin was determined in a Model E analytic ultracentrifuge at 20°C utilizing low-speed sedimentation equilibrium with interference optics. A six-hole centerpiece made possible simultaneous molecular weight determination on untreated and methyldopa-treated gamma globulin. Rotor speeds ranged from 3200 to 4200 rpm. An overspeeding program was employed to shorten the attainment of equilibrium which was reached within 24 hr. Protein concentrations were determined by synthetic boundary techniques. Data were recorded on Kodak II-G plates, and evaluated by the "fringe-trace" method using the Nikon VI-C Comparator. The apparent molecular weight at any given protein concentration was evaluated from the change in the natural log of concentration (ln J) with respect to the change in radius in sq cm ($r^2$) by application of the Svedberg equation.

**RESULTS**

**Induction of Positive Indirect Antiglobulin Reactions**

All sera or gamma globulin solutions to which methyl dopa was added darkened on standing. Positive antiglobulin reactions up to 2+ were obtained 2–4 days after adding...
methyldopa to serum. With purified gamma globulin positive results were obtained by incubation for 16 hr at 25°C, or by incubation at 37°C for the times specified in Table 1. The addition of increasing amounts of albumin to the reaction mixture inhibited the development of positive indirect antiglobulin reactions (Table 2). This inhibition was only in part due to interference by albumin with the reaction between methyldopa and gamma globulin, since albumin also decreased antiglobulin positivity when it was used to dilute the methyldopa-treated gamma globulin after incubation.

Extensive dialysis of methyldopa-treated serum or gamma globulin did not alter development of antiglobulin positivity of red cells subsequently incubated in these solutions.

Comparable results were obtained when equimolar concentrations of hydroquinone were substituted for methyldopa. The addition of ascorbate inhibited the development of positive antiglobulin reactions, as well as the darkening observed in protein solutions which contained hydroquinone.

Latex beads and Rh null cells were also agglutinated by antiglobulin sera following incubation with methyldopa-treated gamma globulin or sera. The titers obtained employing latex beads were in close agreement with those obtained with red cells. Serum or gamma globulin treated in the same manner except for the omission of methyldopa gave negative results.

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**Table 1. Development of Indirect Antiglobulin Reactivity as a Function of Time of Incubation and Methyl Dopa Concentration**

<table>
<thead>
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<th>mg/ml Aldomet</th>
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*10 mg/ml gamma globulin solution. 0.2-cc aliquots were removed, incubated an additional hour with red cells, washed, and then tested with the antiglobulin reagent.

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**Table 2. Effect of Albumin Concentration on Development of Antiglobulin Reactivity**

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<th>1:3:1</th>
<th>1:1</th>
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*Details as per text and Fig. 1. In this study results were scored as strong (S) and weak (W).
Binding of Methyl Dopa to Gamma Globulin

Gel filtration studies. Gel filtration patterns of $^{14}$C-methyldopa-treated and control gamma globulin solutions are given in Fig. 1. The methyldopa-treated gamma globulin is eluted more rapidly from the column than is untreated gamma globulin. Radioactivity is localized over the more rapidly eluting fraction of methyldopa-treated gamma globulin.

When $^{14}$C-methyldopa-treated gamma globulin was dialyzed against phosphate buffered $8\ M$ urea and then gel filtered in phosphate-buffered urea, the radioactivity eluted remained associated with the protein fraction, indicating covalent linkage of methyldopa to the protein.

Dialysis studies. After 24 hr of incubation, approximately 25% of the $^{14}$C-methyldopa was bound to gamma globulin, as measured by dialysis to equilibrium in borate buffer. The actual figures were 25.8% when the protein was counted and 28.2% when the dialysis fluid was counted. When urea-borate was employed as the dialysis medium and dialysand and dialysate employed for calculation, 29.1% and 28.7%, respectively, of methyldopa was found to be protein bound. Greater than 98% of the initial radioactivity was recovered in each experiment.

Trichloroacetic acid precipitation studies. Similar results were obtained when gamma globulin binding of methyl dopa was studied by trichloroacetic acid precipitation of protein from incubation mixtures of $^{14}$C-methyldopa and gamma globulin (Fig. 2). Approximately 30% of the $^{14}$C-methyldopa was found to be trichloroacetic acid precipitatable at 24 hr.

Binding to Gamma Globulin Fragments

When Fab and Fc fragments of methyldopa-treated gamma globulin were isolated, 33.4% of $^{14}$C-methyldopa was bound to gamma globulin at 24 hr. 68.2% and 31.8% of
the protein-bound isotope were found associated with Fab and Fc fragments, respectively. 83% of the initial radioactivity was accounted for after separation of the gamma globulin fragments.

**Determination of Apparent Change in Protein Concentration by Sedimentation Velocity**

Sedimentation velocity experiments demonstrate that mass is lost from the methyl-dopa-treated gamma globulin peak as compared to the untreated gamma globulin solution (Fig. 3). Loss of mass was found to be a function of the concentration of methyl-dopa employed, temperature, and time of incubation. Addition of sodium ascorbate decreased the loss of mass. No new sedimenting peaks appeared during the course of these investigations, but considerable protein accumulated at the bottom of the centrifuge cells containing methyl-dopa-treated protein.

**The Effect of Methyl-dopa Upon Molecular Weight of Gamma Globulin**

Figure 4 shows the results of low-speed equilibrium experiments of solutions containing 10 mg/ml gamma globulin and 0.025 mg/ml methyl-dopa incubated for 16 and
Fig. 4. Aggregation of gamma globulin by methyl dopa demonstrated by low-speed equilibrium centrifugation. Protein concentration in fringes (J) is shown at equilibrium as a function of distance from the axis of rotation in sq cm. The extrapolated line for the apparent lowest-molecular-weight species in the centrifuge cell is given in each case by the dotted line. Representative apparent molecular weights for the protein aggregates within the cell are also given. Results are shown for (A) a gamma globulin solution after 40 hr at 25°C (top), (B) after 18 hr (middle), and (C) after 40 hr, of incubation with methyl-dopa at 25°C. Rotor speed was 4000 rpm at 20°C. Gamma globulin (10 mg/ml) and methyl-dopa (0.025 mg/ml) were incubated for 25°C and then dialyzed against phosphate-buffered saline for 18 hr prior to introduction into the centrifuge cell.
Fig. 5. Inhibition of methyldopa-induced protein aggregation by ascorbate. Low-speed equilibrium centrifugation. Gamma globulin distribution shown at equilibrium for (A) methyldopa (0.025 mg/ml) treated gamma globulin, (B) untreated gamma globulin, (C) ascorbate (0.025 mg/ml) and methyldopa (0.025 mg/ml) treated gamma globulin.

40 hr at 25°C. The 40-hr untreated gamma globulin control (Fig. 4A) is the most aggregated control, but most of the gamma globulin had an apparent molecular weight of 165,000. In sharp contrast, after 18 hr of incubation with methyldopa (Fig. 4B) more marked molecular weight heterogeneity is demonstrated by the curvilinear plot. After the second day of incubation (Fig. 4C), even higher-molecular-weight aggregates are seen. Nevertheless, more protein is present at molecular weight close to the molecular weight of native gamma globulin due to precipitation of the higher-molecular-weight aggregates. The inhibition of methyldopa-induced aggregation of gamma globulin by ascorbate is shown in Fig. 5.

DISCUSSION

These studies demonstrate that serologically detectable methyldopa-treated gamma globulin is fixed to red cells. During incubation of methyldopa with gamma globulin, the drug or one of its metabolites is covalently bound to the gamma globulin. This results in structural modification and aggregation of the gamma globulin which is presumably responsible for both the adherence and serologic detectability of gamma globulin on the red cell. The role of the erythrocyte, as a passive carrier particle for methyldopa-aggregated gamma globulin, is illustrated by the reaction with latex beads. The extent of gamma globulin aggregation by methyldopa is dependent on the concentration of methyldopa, and the time and temperature of its incubation with protein. The solutions of gamma globulin and methyldopa turn brown, suggesting oxidative change in the methyldopa. Both the serological findings and protein aggregation may be inhibited by the addition of a reducing agent, i.e., ascorbate, to the incubation mixture.

A proposed mechanism for the reactions involved is shown in Fig. 6.13,14 Hydroquinones are readily oxidized to the highly reactive, colored quinones. This reaction occurs spontaneously but may be enzymatically catalyzed, i.e., tyrosinase.13,14 A variety of substituted hydroquinones, including aminophenols and heterocyclic compounds, are also readily oxidized.13-15 In the course of autoxidation of hydroquinones, hydrogen peroxide is formed.14 This product is not usually encountered when the
reaction is enzymatically catalyzed. The polyvalent quinones then may react covalently with exposed amino or sulphydryl groups of proteins, and function as a true protein cross-linking agent. This mechanism is responsible for the hardening of the exoskeleton of crustacea, the development of the cuticles of the arthropods, and the formation of melanin and its close association with protein, and is used in leather tanning. In addition, skin sensitization readily occurs with dinitrophenol analogs capable of covalently interacting with proteins. Ascorbate and other reducing agents prevent the oxidation of the hydroquinone to the resonant, highly reactive quinone. The presence of reducing agents which may be added to commercially available methyldopa to prevent oxidation would have a similar effect.

Thus the findings of Wurzel and Silverman using washed red cells almost certainly are a consequence of the interaction of quinone and gamma globulin which was adherent to the red cell membrane. As a result, the cells were rendered directly antiglobulin positive.

The induction of heterospecific antibodies are similarly explicable as resulting from the interaction of methyldopa and plasma proteins. The antibodies produced would consequently be directed toward these altered or denatured proteins. The heterospecific nature of these antibodies might, indeed, be anticipated from studies which indicate that protein may be made heterospecifically antigenic by virtue of exposure of new antigenic sites during denaturation and that the means of denaturation is important in both the frequency and type of sensitization which may develop. Although rheumatoid factor has been reported in a number of patients taking methyldopa, a study detailing the incidence of antibodies to allotypic and heterospecific gamma globulin has yet to be made.

Lobuglio and Jandl obtained negative results when red cells were incubated with 1-5 mM methyldopa. When sera from patients with positive direct antiglobulin reactions due to methyldopa were incubated with methyldopa (1-10 mM) prior to incuba-
tion with normal red cells, negative results were again obtained. The time of incubation of drug, red cells, and sera employed was, however, considerably shorter than those used in either this or the preceding study. At higher concentrations of drug, "non-specific" binding of protein to red cell was observed.

The mechanisms by which drugs may induce hemolytic anemia or antiglobulin positivity have been the subject of a number of excellent reviews. Unlike the mechanisms proposed for the "penicillin," "innocent bystander," or "cephalothin" types of erythrocyte sensitization, the mechanism proposed for the methyldopa phenomenon must account for the fact that neither the drug nor its metabolites can be shown to be necessary for the in vitro development of red cell sensitization, and that hapten inhibition cannot be demonstrated.

In addition, the substances coating the red cell membrane in the methyldopa-sensitized erythrocyte have been reported to have a high degree of specificity for the Rh locus. Nonetheless, some question has developed regarding the nature of the Rh specificity of the eluates obtained. Neither we nor others (RE Rosenfeld, personal communication) have been able to identify such specificity. The eluates, moreover, appear to be fully reactive with Rh null cells (RE Rosenfeld, personal communication).

In the "fluid mosaic model" of membrane structure, the Rh locus is visualized as a globular glycopolypeptide chain floating on a sea of membrane lipid and phospholipid. Distribution of the D site appears governed by the thermodynamics of the interaction of protein and lipid. In Rh null cells these integral glycoprotein components are apparently deleted, and a significant reorientation of the charge and topography of the membrane would result. Thus, Rh null individuals have structurally deformed cells with decreased red cell survival. On the basis of charge and topographic considerations, it might be mistaken to confer antibody specificity solely on the basis of failure to react with this marred membrane. Further, all gamma globulin reacting with the highly active sites and structures on the red cell membrane need not perforce be antibody gamma globulin.

In vivo, significant amounts of nonantibody gamma globulin as well as other plasma proteins are bound to the red cell membrane and are maintained in slow equilibrium with the plasma proteins. In vitro, it may be shown that the quantity of gamma globulin bound is dependent on the gamma globulin concentration of the media. At high gamma globulin concentration, the number of molecules antibody gamma globulin and nonantibody gamma globulin bound are similar. The "nonspecific" bond appears quite firm; the exchange rate from red cell membrane to media is similar for specifically and nonspecifically bound gamma globulin, and there appears to be a finite member of binding sites for both antibody and nonantibody protein. These similarities are evident whether intact gamma globulin or papain fragments of gamma globulin are employed. Unlike the absorption of antibody globulin, however, a competition of binding sites exists between albumin and nonantibody gamma globulin. Most importantly, even under conditions where similar number of molecules are bound to render specifically sensitized cells strongly antiglobulin positive, the antiglobulin reaction consistently fails to detect nonspecifically absorbed material.

Heat-aggregated nonantibody globulin displays even greater affinity than native gamma globulin for the erythrocyte membrane. Although in the current study methyldopa-treated gamma globulin proved detectable by serologic methods while
native gamma globulin was not, we have no information as to its detectability or binding affinity as compared to gamma globulin denatured by other mechanisms.

An alternative mechanism for antiglobulin sensitization in patients receiving methyldopa or its structural congeners can therefore be proposed. In this view, antiglobulin sensitization occurs as a result of the uptake of serologically detectable altered gamma globulin on the red cell surface (Fig. 7). Although not reported in the current study, sensitization can be obtained in vitro at levels of methyldopa as low as 5 μg/ml. This concentration is close to the therapeutic concentration of methyldopa in vitro and less than what might be obtained in the portal vein during active absorption of the drug from the gastrointestinal tract. In this view, the erythrocyte would once again serve as an “innocent bystander” in its absorption of denatured material. This mechanism would account for the dose–time relationship necessary for development of red cell sensitization as well as the lack of an anamnestic response when methyldopa is readministered to previously “sensitive” individuals. A similar mechanism, involving the interaction of benzoquinone acetic acid, a metabolite of homogentisic acid, has been proposed to account for the darkening and destruction of connective tissue in alcaptonuria. One would also anticipate that structural analogs of methyldopa, such as L-dopa, would cause red cell sensitization without a high incidence of overt hemolysis and that an increasing incidence of red cell sensitization would occur when increased doses of drug are administered.

Available data suggest that in the rare individual with overt hemolysis true circulating antibodies are present. Whether these antibodies result from the alteration of red cell membrane protein by methyldopa or represent an intercurrent autoimmune hemolytic anemia is problematic.

Clearly, an extension of our in vitro observations would be at variance with the concept of specificity of the red cell eluates and of the formation of true autoantibodies which reportedly occurs in patients receiving methyldopa. If true antibodies are indeed induced a chemical alteration of naturally occurring red cell antigens with induced antibody formation would nonetheless appear a more likely mechanism than those proposed previously (Fig. 7). The resultant phenomenon would thus be immune rather than “autoimmune.”

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