

# Suppression of In Vitro Monoclonal Human Rheumatoid Factor Synthesis by Antiidiotypic Antibody

## TARGET CELLS AND MOLECULAR REQUIREMENTS

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**ABSTRACT** Previous studies have indicated that antiidiotypic antibody can modulate expression of idiotype both in vivo and in vitro. Although the precise mechanisms underlying modulation of idiotype expression by antiidiotype remains unclear, a requirement for intact IgG antiidiotypic antibody has been suggested and T cells appear to play a role in some systems. We have studied peripheral blood mononuclear leukocytes (MNL) from a patient with a B cell lymphoma and a circulating IgM $\kappa$  rheumatoid factor (RF) paraprotein in an effort to delineate mechanisms involved in regulation of idiotype expression by antiidiotypic antibody. 1–10% of MNL from this patient could be cytoplasmically stained with specific F(ab')<sub>2</sub> antiidiotypic antibody. MNL from the patient spontaneously synthesized IgM RF in culture that possessed the same idiotype as the circulating IgM RF paraprotein. Production of RF by MNL was suppressed by pretreatment with either intact IgG or the F(ab')<sub>2</sub> fragments of antiidiotypic antibody (50% inhibitory concentration was 0.2 and 1.1  $\mu$ g/culture, respectively). In contrast, the Fab' fragment of antiidiotypic antibody was not inhibitory (up to 57  $\mu$ g/culture) despite retaining demonstrable antiidiotype activity. Suppression of RF production was not observed over the same concentration range with the IgG or F(ab')<sub>2</sub> fractions of a non-cross-reactive antiidiotypic antibody prepared against another monoclonal IgM $\kappa$  RF paraprotein or with IgG or F(ab')<sub>2</sub> fractions prepared from

normal rabbit serum. Inhibition of RF production by antiidiotypic antibody did not require T cells. Antiidiotypic antibody decreased intracellular and extracellular levels of idiotype indicating diminished synthesis of idiotype by the patient's B cells. Synthesis of IgM RF by MNL obtained from unrelated donors was not suppressed by the antiidiotypic antibody specific for the patient's paraprotein. The results indicate that (a) antiidiotypic antibody is capable of directly suppressing human B cell release of idiotype, (b) the bivalent antigen-binding fragment (F[ab']<sub>2</sub>) of antiidiotypic antibody is sufficient for mediating such suppression, (c) an intact Fc portion of antiidiotypic antibody enhances suppression of idiotype, and (d) antiidiotypic antibody inhibits idiotype expression by suppressing synthesis of idiotype.

## INTRODUCTION

Interactions between idiotype and antiidiotype appear to play a role in regulation of immune responses, as initially suggested by Jerne (1). These interactions can occur at either a cellular level (2–4) or by way of humoral pathways, such as reactions of antiidiotypic antibody with cell-surface idiotype (5–8). In the latter case, both in vitro and in vivo studies have demonstrated that antiidiotypic antibody is capable of modulating subsequent expression of idiotype. The seemingly paradoxical capacity of antiidiotypic antibody to either induce (9–15) or suppress (5–8) expression of idiotype is consistent with other observations that interactions between idiotype and antiidiotype are demonstrable in both helper (3) and suppressor (2) regulatory T cell circuits. Despite evidence that antiidiotypic antibody is capable of potentially regulating levels

Portions of this work were presented in part at the Annual Meeting of the American Society of Clinical Investigation, May 1982, Washington, DC.

Received for publication 14 September 1982 and in revised form 22 June 1983.

of idiotype, mechanisms underlying the effects of anti-idiotypic antibody remain unclear. In this regard, previous *in vivo* (16) and *in vitro* (17) studies in mice have suggested that an intact Fc portion of anti-idiotypic IgG is required for antibody-induced suppression of idiotype expression.

In this study, we analyzed the effects of rigorously characterized anti-idiotypic antibody preparations on *in vitro* release of idiotype by peripheral blood lymphocytes obtained from a patient with a monoclonal B cell lymphoma. The data indicate that (a) anti-idiotypic antibody is capable of directly suppressing human B cell release of idiotype, (b) the bivalent antigen-binding fragment (F[ab']<sub>2</sub>) of anti-idiotypic antibody is sufficient for mediating such suppression, (c) an intact Fc portion of anti-idiotypic antibody enhances suppression of idiotype, and (d) anti-idiotypic antibody inhibits idiotype expression by suppressing synthesis of idiotype.

## METHODS

**Patient.** The patient (Mil) is a 47-yr-old white female who was diagnosed as having a B cell lymphoma on the basis of lymph-node biopsy. Serum protein electrophoresis and immunoelectrophoresis revealed the concomitant presence of a monoclonal IgM $\kappa$  paraprotein (consistently >4.0 g/dl) possessing rheumatoid factor (RF)<sup>1</sup> activity as assessed by the latex-fixation test (18) and solid-phase radioimmunoassay (RIA) (19). During the course of this study, the patient was intermittently treated with prednisone and chlorambucil, but blood was also obtained from the patient in the absence of any therapy and the results were comparable to those obtained on chemotherapy.

**Preparation and culture of peripheral blood mononuclear leukocytes (MNL).** MNL were prepared by density centrifugation according to the method of Böyum (20). The cells were washed two times in minimal essential medium, five times in RPMI 1640 containing 5% heat-inactivated fetal calf serum (Microbiological Associates, Walkersville, MD), and two times in complete medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum, 2 mM glutamine, and 20  $\mu$ g/ml gentamycin). The cells were adjusted to  $2 \times 10^5$  cells/ml and cultured in 1.0-ml aliquots in plastic tubes in the presence of varying concentrations of the anti-idiotypic antibody preparations for 2 d at 37°C in a 5% CO<sub>2</sub> and air atmosphere. The cells were then centrifuged (1,700 rpm for 10 min), washed, resuspended in complete medium, and cultured under the same conditions for 5 d. At the termination of culture, the tubes were centrifuged and culture supernatants harvested and frozen at -20°C until assayed for IgM RF and IgM.

For quantitation of intracellular IgM and IgM RF, single cell suspensions were washed three times in phosphate-buffered saline (PBS) containing 1% fetal calf serum, resuspended at  $5 \times 10^6$  cells/ml and disrupted by sonication at

0°C for 1 min at 20,000 cycles/s at 90 W (Sonifer Cell Disruptor model W140, Heat Systems-Ultrasonics, Inc., Plainview, NY), as described by Kutteh et al. (21). The sonicate was clarified by centrifugation for 5 min at 12,800 g in an Eppendorf microcentrifuge (Sybron Corp., Medical Products Div., Westbury, NY) before RIA.

**Isolation of T cell and enriched B cell fractions.** T cells and enriched B cells were isolated from MNL by the method of Saxon et al. (22). Briefly, MNL were rosetted with sheep erythrocytes (SRBC) treated with 2-amino-ethylisothiuronium bromide hydrobromide at 37°C for 10 min and then at 4°C for 20–30 min after centrifugation (1,000 rpm for 10 min). The mixture was gently layered on Ficoll-Hypaque (Pharmacia Fine Chemicals, Inc., Piscataway, NJ) and the rosetted cells separated from nonrosetting cells by density gradient centrifugation. Both rosetting and nonrosetting fractions were then re-rosetted before an additional density gradient centrifugation over Ficoll-Hypaque to obtain further purification. SRBC in the T cell fraction were lysed with a KHCO<sub>3</sub>-buffered ammonium chloride solution. As previously described (23), this method in our hands consistently yields a T cell population consisting of <3% nonrosetting cells and  $\leq 1\%$  surface Ig-positive cells and a B enriched population consisting of 45–60% surface Ig-positive cells and <2% rosetting cells.

**RIA of IgM RF.** The procedure utilized in these studies has been reported in detail (19). Briefly, triplicate aliquots of the patient's isolated monoclonal IgM RF (mRF) (dose range 0.25–25 ng/well) or unknown samples were placed into polystyrene microtiter wells (Dynatech Laboratories Inc., Alexandria, VA) coated with human IgG and wells coated with RIA grade bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO) as a control for nonspecific binding. After an 18–20-h incubation at room temperature, the wells were aspirated, washed three times with veronal-buffered saline (VBS) containing 1% BSA (VBS-BSA) and incubated with 0.25 ml VBS-BSA containing 10 ng of <sup>125</sup>I-labeled affinity-purified goat anti-human IgM. Standard curves were constructed for each experiment by plotting mean counts per minute <sup>125</sup>I-labeled anti-IgM bound (IgG well mean minus BSA well mean) for each dilution of unknown and then referring to the standard curve. All assays were performed in duplicate or triplicate at two dilutions. The assay has a coefficient of variation between 8.2 and 18.6%, depending on the concentration of IgM RF in the sample.

**RIA of IgM.** Total IgM in culture supernatants was quantitated by solid-phase RIA by a modification of previously reported methods (24). Microtiter wells were precoated with 1  $\mu$ g/well of the IgG fraction of specific goat anti-human IgM. Standards for each assay consisted of the patient's isolated IgM RF paraprotein and a reference normal human serum containing  $1.22 \pm 0.25$  mg/ml of IgM as determined by a fluorometric immunoassay (FIAX, International Diagnostic Technology, Santa Clara, CA). Unknowns and standards were incubated overnight at room temperature in the precoated microtiter wells and then aspirated and washed three times with VBS-BSA. After the final wash, 10 ng <sup>125</sup>I-labeled affinity purified goat anti-human IgM was added to each well and incubated overnight at room temperature. The wells were then aspirated and washed three times with VBS-BSA. Individual wells were placed in counting vials and counted for 1 min in a Beckman 4000 gamma counter (Beckman Instruments, Inc., Palo Alto, CA). Standards were assayed in triplicate and unknowns in duplicate at two dilutions. Background binding was always <3% of the radiolabeled antibody added. Standard curves were constructed for

<sup>1</sup> Abbreviations used in this paper: MNL, mononuclear leukocytes; mRF, monoclonal rheumatoid factor; RF, rheumatoid factor; SRBC, sheep erythrocytes; TRITC, tetramethyl rhodamine isothiocyanate; VBS, veronal-buffered saline.

each experiment by plotting counts per minute of  $^{125}\text{I}$ -labeled anti-IgM bound vs. input of standard IgM. Values for unknowns were calculated from the standard curve. Specificity controls performed for each RIA consisted of testing excess quantities (up to 1  $\mu\text{g}$ /well) of IgG and IgA for interference in the RIA. No interference was observed, confirming the specificity of the technique utilized.

**Isolation of RF.** Since IgM $\kappa^{\text{Mil}}$  from patient Mil (IgM $\kappa^{\text{Mil}}$ ) did not precipitate as a euglobulin, the paraprotein was initially separated from other serum proteins by precipitation in one-half saturated ammonium sulfate and further purified by repeated gel filtration through Sephacryl S-300 in 0.1 M sodium acetate buffer, pH 4.1. The product was free of other serum proteins when examined by immunoelectrophoresis with antiserum to normal human serum. Ouchterlony analysis with specific antisera revealed only a trace of IgA in addition to the IgM protein.

Monoclonal IgM $\kappa^{\text{Slo}}$  possessing RF activity was precipitated from the serum of patient Slo as a mixed cryoglobulin. After washing twice with cold distilled water, the cryoprecipitate was dissolved in 0.1 M sodium acetate buffer, pH 4.1, and fractionated into its components by gel filtration through Sephacryl S-300 in the same buffer. The isolated IgM $\kappa^{\text{Slo}}$  was free of IgG and IgA by Ouchterlony analysis with specific antisera and was free of other serum proteins by immunoelectrophoresis with antiserum to normal human serum.

**Antidiotypic antisera.** Antiserum to IgM $\kappa^{\text{Mil}}$  was raised in an albino rabbit by subcutaneous injection of the purified paraprotein (2 mg) emulsified in complete Freund's adjuvant, followed 1 wk later by three sequential injections of 0.5 mg of the protein emulsified in incomplete adjuvant at weekly intervals. The animal was bled one week after the last injection. Rabbit antiserum to IgM $\kappa^{\text{Slo}}$  was produced by a similar immunization schedule.

Antisera were rendered specific for idiotypic determinants by absorption with normal human serum proteins covalently conjugated to CNBr-activated agarose gel (25). 2-ml aliquots of the antisera were diluted with an equal volume of 0.15 M sodium chloride, 0.02 M sodium phosphate, pH 7.4 (PBS), and passed twice through a 5-ml column of the immunoadsorbent containing ~25 mg of covalently bonded protein. Removal of antibodies to shared determinants of IgM was further insured by passage twice through a 5-ml agarose gel column containing 20 mg of covalently bound monoclonal IgM $\kappa^{\text{Dau}}$  protein, which does not possess RF activity.

To verify the specificity of the absorbed antisera, the capacity to bind the immunogen and an unrelated monoclonal IgM protein of the same light chain type (IgM $\kappa^{\text{Dau}}$ ) was examined before and after absorption by a two-step direct-binding radioassay. All solutions were prepared in PBS containing 0.1% RIA grade BSA. Equal aliquots (0.1 ml) of antiserum dilution, the purified paraprotein IgM $\kappa^{\text{Mil}}$  or IgM $\kappa^{\text{Dau}}$  (100 ng/ml) labeled with  $^{125}\text{I}$  (26) (2,000–10,000 cpm/ng) and 0.05 M dithiothreitol were mixed and reacted at room temperature for 16 h. The dithiothreitol prevented interference due to the interaction of IgG with RF active sites by dissociating the IgM into 7S subunits, but did not prevent their subsequent reaction with anti-IgM or antidiotypic antibodies. Antibody-bound  $^{125}\text{I}$ -IgM was separated from free antigen by adsorption to killed *Staphylococcus aureus* (Pansorbin, Calbiochem-Behring Corp., San Diego, CA) added as a 1% suspension (0.2 ml). After reacting for 1 h, the adsorbent was recovered by centrifugation, washed three times with PBS, and counted in a gamma counter (Biogamma, Beckman Instruments, Inc.).

**IgG, F(ab')<sub>2</sub>, and Fab' antidiotypic preparations.** The

IgG fractions of the absorbed antisera were isolated by precipitation with half-saturated ammonium sulfate followed by gel filtration through Sephacryl S-300 in 0.08 M sodium phosphate buffer, pH 7.5. The resulting preparations yielded single bands of the appropriate mobility by polyacrylamide gel electrophoresis in 0.1 M sodium phosphate buffer, pH 7.0, containing 0.1% sodium dodecyl sulfate (SDS) (27). The IgG fraction of antidiotypic antiserum to IgM $\kappa^{\text{Mil}}$  was further examined by sedimentation-equilibrium analysis in an analytical ultracentrifuge equipped with photoelectric scanner absorption optics (28). These latter studies confirmed the homogeneity of the preparation and indicated a mol wt of 143,400, assuming a partial specific volume ( $\bar{V}$ ) of 0.738 ml/g (29).

F(ab')<sub>2</sub> fragments of the antidiotypic antibodies were prepared by digestion of the globulin fraction of absorbed antisera with pepsin in 0.1 M sodium acetate buffer, pH 4.5, at 37°C for 16 h (30). The substrate to enzyme ratio was 100:1 by weight. After neutralizing with Tris base or dialysis into 0.08 M sodium phosphate buffer, pH 7.5, the F(ab')<sub>2</sub> fragments were isolated by gel filtration through Sephadex G-200 or Sephacryl S-200 in the pH-7.5 phosphate buffer. The identity and homogeneity of the isolated fragments were verified by polyacrylamide gel electrophoresis in SDS-phosphate buffer. The F(ab')<sub>2</sub> fragments of the anti-IgM $\kappa^{\text{Mil}}$  antibodies were also characterized by sedimentation-equilibrium analysis in the analytical ultracentrifuge. These studies indicated the presence of a single component having a mol wt of 97,400, assuming the  $\bar{V}$  to be equal to that of the unfragmented IgG molecule.

Fab' fragments of antidiotypic antibodies were prepared by digestion of the globulin fraction of the absorbed antiserum with pepsin in pH-4.5 sodium acetate buffer for 16 h in the presence of 0.01 M cysteine (30). Digestion was terminated by dialysis into 0.08 M sodium phosphate buffer, pH 7.5, containing 0.01 M iodoacetamide and the Fab' fragments isolated by gel filtration through Sephacryl S-200. Polyacrylamide gel electrophoresis in SDS-phosphate buffer also verified that cleavage had proceeded to the monomeric Fab' fragment and further demonstrated the absence of other cleavage products. Furthermore, the isolated Fab' fragments of the anti-IgM $\kappa^{\text{Mil}}$  antibodies appeared homogeneous by sedimentation-equilibrium ultracentrifugal analysis and demonstrated a mol wt of 46,600, assuming  $\bar{V}$  to be equal to that of the rabbit IgG.

Normal rabbit IgG (Sigma Chemical Co.) was further purified by gel filtration through Sephacryl S-300 in 0.08 M sodium phosphate buffer, pH 7.5. F(ab')<sub>2</sub> and Fab' fragments of the normal IgG were prepared as described for antibody preparations. Each gave a single band of the expected mobility by polyacrylamide gel electrophoresis in SDS-phosphate buffer and was homogeneous by sedimentation-equilibrium in the ultracentrifuge. Molecular weights of the normal rabbit IgG, F(ab')<sub>2</sub>, and Fab' preparations calculated from the latter analyses were 143,500, 98,300 and 45,100, respectively.

**Anti- $\mu$  antiserum.** Rabbit anti- $\mu$  antiserum was the kind gift of Drs. Hiromi Kubagawa and Max Cooper, University of Alabama in Birmingham. Before use, the antiserum was passed through agarose gel columns containing covalently bound pooled human IgG and human monoclonal IgA1. The IgG fraction of anti- $\mu$  was isolated by precipitation of the absorbed antiserum with half-saturated ammonium sulfate and gel filtration through Sephacryl S-300 as described for the antidiotypic preparations.

**Assay for antidiotypic activity.** Antidiotypic activity of antibody preparations was assessed by their capacity to

inhibit reaction of the appropriate mRF in the solid-phase RIA for IgM RF. The assay was performed in polystyrene microtiter wells coated with 0.3 ml of DEAE-cellulose purified human IgG (50  $\mu$ g/ml) in PBS for 1 h at room temperature. The wells were then washed three times with PBS and the remaining protein binding sites blocked by incubation with 0.4 ml of 0.1% RIA grade BSA in PBS. The wells were then washed an additional three times with PBS before use. A parallel set of wells was prepared in an identical fashion except that the initial incubation with IgG was omitted (BSA control wells). PBS containing 1% RIA grade BSA (PBS-BSA) served as the diluent for all solutions used in the assay. Equal volumes of diluted antibody and mRF (100 ng/ml) were mixed and duplicate 0.2-ml aliquots were placed in both the IgG-coated wells and BSA control wells. Total activity controls prepared by substituting diluent alone for the antibody dilution were included in each assay. After incubating 16 h at room temperature, the wells were aspirated, washed three times with PBS, and incubated an additional 4 h with 0.2 ml PBS-BSA containing 10 ng  $^{125}$ I-labeled anti-IgM/well. The wells were again aspirated and washed, then counted for 1 min in a gamma counter (Biogamma, Beckman Instruments, Inc.). Results obtained with the IgG wells were corrected for nonspecific binding of the mRF as determined by the simultaneous analysis of the inhibition mixtures in the BSA control wells, and for background binding of the  $^{125}$ I-labeled anti-IgM as determined by analysis of diluent alone. These corrections were small in all instances, being <0.5% of the counts added for the former and <1% of the counts added for the latter. Percentage of inhibition was calculated by comparison to the total activity control:

Percentage of inhibition

$$= 100 \times \left( 1 - \frac{\text{counts per minute of inhibition mixture}}{\text{counts per minute of total activity control}} \right)$$

**Immunofluorescence studies.** Peripheral blood MNL from the patient were examined for the presence of B cells cytoplasmically staining with F(ab')<sub>2</sub> preparations of anti-idiotypic antibody (31). Fixed cytocentrifuge preparations of MNL were incubated with F(ab')<sub>2</sub> preparations of anti-idiotypic antibody or control F(ab')<sub>2</sub> prepared from normal rabbit IgG for 45 min at room temperature and washed for 2 h in PBS. The slides were then incubated with tetramethyl rhodamine isothiocyanate (TRITC)-labeled goat anti-rabbit F(ab')<sub>2</sub> (a kind gift of Dr. Jiri Mestecky, University of Alabama in Birmingham), washed in PBS, and examined with a fluorescence microscope (Orthoplan, E. Leitz Inc., Rockleigh, NJ) equipped with a vertical illuminator (Ploempak 2, E. Leitz Inc.) containing filter module N with a super pressure mercury lamp (HBO 100W/2, Osram Sales Corp., Siemens Corp., W. Germany) as light source for optimal TRITC excitation. Specificity controls were performed by mixing the F(ab')<sub>2</sub> anti-idiotypic with excess idiotype (IgM<sup>Mil</sup>) or an unrelated mRF protein (IgM<sup>Slo</sup>) before addition to cells. Only excess idiotype blocked binding of the anti-idiotypic to the patient's MNL. Furthermore, TRITC-labeled goat anti-rabbit F(ab')<sub>2</sub> did not stain the patient's MNL.

Cells exhibiting surface idiotype were detected by incubating the patient's MNL with F(ab')<sub>2</sub> anti-idiotypic (or F(ab')<sub>2</sub> prepared from normal rabbit IgG) at 4°C in PBS containing 0.1% sodium azide (31). After 45 min of incubation, the cells were washed with PBS at 4°C and then incubated with TRITC-labeled goat anti-rabbit F(ab')<sub>2</sub> at 4°C for 45 min. After washing, the cells were immediately examined for surface staining. Controls were performed as described above for cytoplasmic staining procedures.

## RESULTS

**Specificity of antiidiotypic reagents.** The mRF paraprotein (IgM<sup>Mil</sup>) was isolated from the patient's serum and a rabbit antiidiotypic serum prepared. As a control for subsequent studies, antiidiotypic serum was prepared against another IgM $\kappa$  RF (IgM<sup>Slo</sup>) in a similar fashion. The specificity of the two antiidiotypic reagents was initially examined in a direct-binding assay. Results obtained with a representative preparation of the antiserum to IgM<sup>Mil</sup> are shown in Fig. 1. Before absorption, detectable quantities of both  $^{125}$ I-IgM<sup>Dau</sup> and  $^{125}$ I-IgM<sup>Mil</sup> were bound by the antiserum when diluted 1:1,600 or greater. After absorption, the globulin fraction (reconstituted to the volume of antiserum taken for absorption) failed to react with the  $^{125}$ I-IgM<sup>Dau</sup> at the lowest dilution tested (1:100), but continued to react with the  $^{125}$ I-IgM<sup>Mil</sup> when diluted 1:1,600. Comparable results were obtained for the antiserum to IgM<sup>Slo</sup> before and after absorption. The individual antiidiotypic specificities of the absorbed antibody preparations were further established by a binding-inhibition assay. Antiidiotypic directed against IgM<sup>Mil</sup> inhibited binding of IgM<sup>Mil</sup> to insolubilized human IgG in a dose-response fashion as depicted in Fig. 2. Insignificant inhibition was observed with antiidiotypic directed against IgM<sup>Slo</sup> (Fig. 2, upper panel). In contrast, anti-IgM<sup>Slo</sup> exhibited dose-related

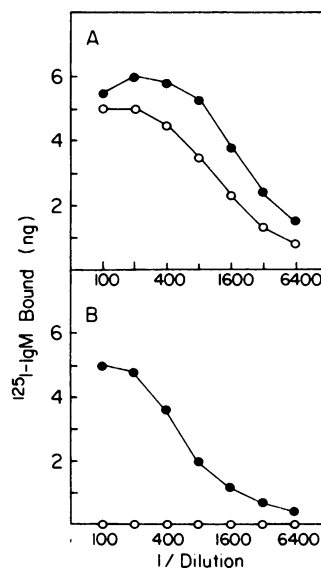


FIGURE 1 Reaction of the antiserum to the mRF from patient Mil with the immunogen (●) and an unrelated IgM protein (IgM<sup>Dau</sup>) (○) as measured by a direct-binding assay (A) before and (B) after absorption to idiotype specificity. The absorbed antiserum was analyzed as the 50% saturated ammonium sulfate precipitated globulin fraction reconstituted to original volume in PBS.

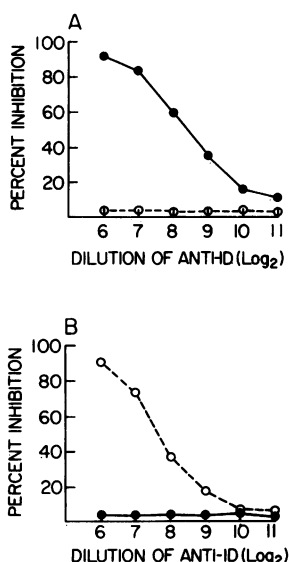


FIGURE 2 Binding-inhibition of the mRF from (A) patient Mil and (B) patient Slo by the IgG fractions of absorbed antiidiotypic antisera specific for IgM $\kappa^{Mil}$  (●) and IgM $\kappa^{Slo}$  (○). The antiidiotypic preparations were each adjusted to 1 mg/ml protein concentration before diluting.

inhibition of the binding of IgM $\kappa^{Slo}$  to human IgG, whereas anti-IgM $\kappa^{Mil}$  was noninhibitory (Fig. 2, lower panel). It should be stressed that the apparent antiidiotypic activity exhibited by the two reagents for their respective idiotypes was comparable. Normal rabbit IgG did not manifest antiidiotypic activity toward either IgM protein (data not shown).

**Preparation of F(ab')<sub>2</sub> and Fab' antiidiotype and analysis of relative antiidiotypic activity.** We next prepared F(ab')<sub>2</sub> and Fab' antiidiotype reagents from the IgG fraction of anti-IgM $\kappa^{Mil}$  and anti-IgM $\kappa^{Slo}$  and assessed the purity of these reagents as indicated in Methods. The preparations were then tested for their capacity to inhibit binding of the relevant mRF to human IgG. As shown in Fig. 3, the F(ab')<sub>2</sub> fraction of anti-IgM $\kappa^{Mil}$  exhibited virtually identical inhibitory activity as the IgG fraction, whereas the Fab' preparation was approximately one-third as active on a weight basis. Neither F(ab')<sub>2</sub> nor Fab' directed against IgM $\kappa^{Slo}$  exhibited any inhibitory activity with regard to the binding of IgM $\kappa^{Mil}$  to human IgG (data not shown). Furthermore, F(ab')<sub>2</sub> and Fab' prepared from normal rabbit IgG were also inactive (data not shown).

Passage of the F(ab')<sub>2</sub> anti-IgM $\kappa^{Mil}$  preparation through a protein A-Sepharose CL-4B (Pharmacia Fine Chemicals) column resulted in no change in the antiidiotypic activity as measured by the binding-inhibition assay, indicating the absence of undigested IgG antibody. Passage of the IgG anti-IgM $\kappa^{Mil}$  prep-

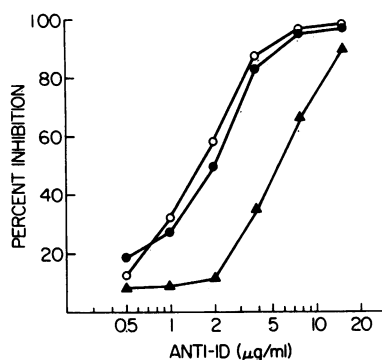


FIGURE 3 Binding-inhibition of IgM $\kappa^{Mil}$  by the IgG (○), F(ab')<sub>2</sub> fragment (●), and Fab' fragment (▲) preparations of the specific antiidiotypic antiserum.

aration through the same column resulted in complete absorption of the antiidiotypic activity. Further evidence that the antiidiotypic activity of the F(ab')<sub>2</sub> preparation was not related to contaminating IgG was provided by the chromatographic studies shown in Fig. 4. IgG (Fig. 4 A) and F(ab')<sub>2</sub> (Fig. 4 B) preparations were passed through a Sephadex G-100 column and the fractions analyzed for protein (absorbance at 280 nm) and antiidiotypic activity by the binding-inhibition assay. In each case, the antiidiotypic activity eluted as a single peak of the appropriate apparent molecular weight and paralleled the absorbance at 280 nm. In addition, analysis of the Fab' anti-IgM $\kappa^{Mil}$  preparation under the same conditions (Fig. 4 C) clearly demonstrated that the antiidiotypic activity in this preparation was not due to residual undegraded IgG or its F(ab')<sub>2</sub> fragments.

**Presence of surface and cytoplasmic idiotype positive cells in patient Mil's peripheral blood.** Peripheral blood MNL obtained from the patient were analyzed for the presence of surface and cytoplasmic idiotype positive cells utilizing F(ab')<sub>2</sub> antiidiotype prepared against the patient's circulating IgM $\kappa$  paraprotein. During the course of this study 1-10% of the patient's MNL cytoplasmically stained with antiidiotype directed against her paraprotein and this staining could be blocked by absorption of the antiidiotype with the patient's paraprotein but not an unrelated mRF (IgM $\kappa^{Slo}$ ). Under the same conditions, staining was not observed with F(ab')<sub>2</sub> antiidiotype directed against IgM $\kappa^{Slo}$  or F(ab')<sub>2</sub> prepared from normal rabbit IgG. Surface idiotype positive cells were also detected in the patient's peripheral blood and were present in comparable numbers to cytoplasmic positive cells.

**Suppression of in vitro release of idiotype by IgG antiidiotypic antibody.** Peripheral blood MNL from the patient were cultured in vitro and observed to spontaneously release IgM RF that possessed the same

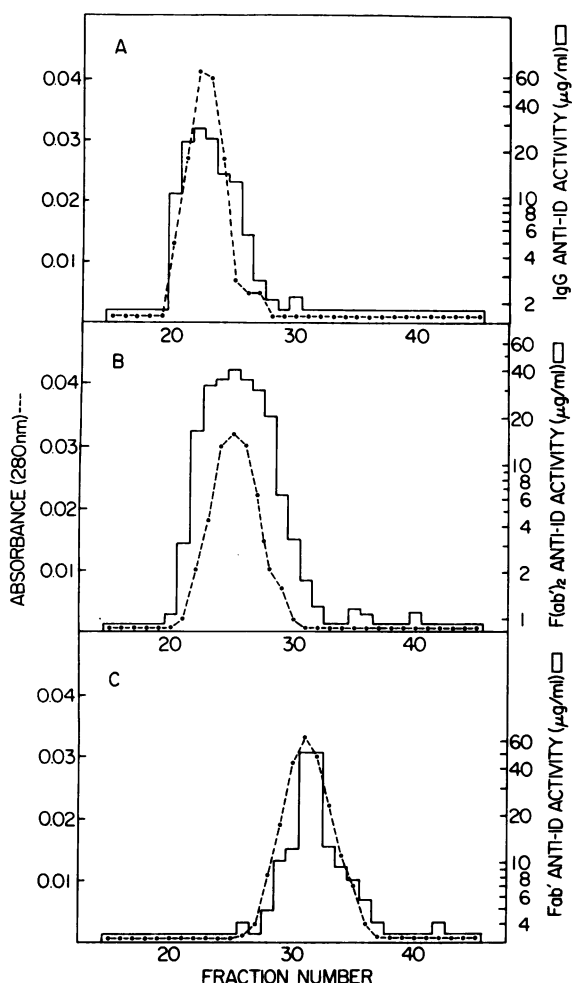


FIGURE 4 Distribution of protein and antiidiotypic activity in the (A) IgG, (B) F(ab)<sub>2</sub> fragment, and (C) Fab' fragment preparations of the antiidiotypic antiserum specific for the mRF from patient Mil by gel filtration. Approximately 100 μg of each preparation was passed through a Sephadex G-100 column (1 × 49 cm) in PBS under identical conditions and the effluent fractions analyzed for protein by absorbance at 280 nm and antiidiotypic activity by the binding-inhibition assay.

idiotypic determinants as the patient's circulating IgM RF paraprotein. In initial experiments, the MNL were cultured either alone or in the presence of varying concentrations of the IgG fraction prepared from anti-IgM<sup>Mil</sup>, anti-IgM<sup>Slo</sup>, or normal rabbit serum. As shown in Fig. 5, only the IgG fraction prepared from the antiserum directed against the patient's circulating IgM paraprotein was inhibitory over the dose range examined (50% inhibitory concentration  $\cong$  0.2 μg/culture). We were concerned that the apparent inhibition of IgM RF release might be related to carry-over of sufficient antiidiotypic antibody from the initial prein-

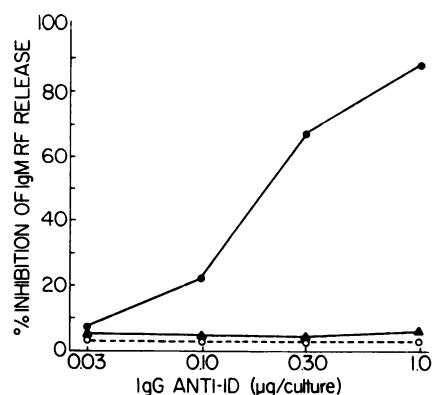


FIGURE 5 Suppression of in vitro IgM RF release by the IgG fraction of antiidiotypic antibody. MNL ( $2 \times 10^5$ /ml) obtained from patient Mil were preincubated for 48 h with varying concentrations of normal rabbit IgG (○), the IgG fraction obtained from an unrelated antidiotypic serum (▲) or the IgG fraction obtained from antiidiotypic serum directed against the patient's IgM RF (●). The cells were then washed and incubated in medium alone for 5 d at 37°C in a 5% CO<sub>2</sub> in air atmosphere. At the termination of culture, cell supernatants were harvested by centrifugation and assayed for IgM RF by solid-phase RIA. Results are expressed as percentage of inhibition of IgM RF release compared with MNL preincubated in medium alone.

cubation to interfere with the activity of IgM RF in the cell supernatants. To assess this possibility, we assayed supernatants for both IgM and IgM RF. Experiments were performed that established that antiidiotypic antibody did not interfere with the IgM RIA for IgM<sup>Mil</sup>. As shown in Table I, identical suppression was observed in both assays, indicating that antiidiotypic antibody suppressed release of IgM RF into the culture fluids. The specificity of this phenomenon was further analyzed by investigating the effect of anti-IgM<sup>Mil</sup> on in vitro pokeweed mitogen-induced synthesis of IgM and IgM RF by peripheral blood MNL obtained from four unrelated normal adult donors. No inhibition of IgM or IgM RF synthesis was observed (data not presented).

To examine further the specificity of the suppression observed with the IgG fraction of antiidiotypic antibody, we compared the inhibitory effects of the IgG fractions prepared from antiidiotypic and anti-μ antisera. Initial experiments indicated that these IgG preparations possessed essentially identical binding activity for IgM<sup>Mil</sup>. In contrast, the IgG fraction prepared from the anti-μ antiserum was <1% as effective in suppressing RF release as the antiidiotypic antibody (results not shown).

*F(ab)<sub>2</sub> but not Fab' antiidiotypic prevents release of idiotype.* We next examined the influence of F(ab)<sub>2</sub> and Fab' preparations obtained from the IgG

TABLE I  
Inhibition of IgM and IgM RF Release by Antiidiotypic Antibody

Reagent	IgM	Inhibition	IgM RF	Inhibition
	ng/culture	%	ng/culture	%
None	275	—	262	—
IgG anti-IgM <sup>Mil</sup> (1.0 µg/culture)	32	88	32	88
IgG anti-IgM <sup>Slo</sup> (1.0 µg/culture)	260	5	265	0
IgG normal rabbit (1.0 µg/culture)	275	0	247	6

fraction of normal rabbit serum, anti-IgM<sup>Mil</sup>, and anti-IgM<sup>Slo</sup>. As shown in Fig. 6, the F(ab')<sub>2</sub> fraction directed against the patient's IgM paraprotein clearly suppressed in vitro release of IgM RF by the patient's MNL (50% inhibitory concentration  $\approx$  1.1 µg/culture), whereas the Fab' anti-IgM<sup>Mil</sup> preparation was not inhibitory at concentrations as high as 57 µg/culture. Furthermore, F(ab')<sub>2</sub> and Fab' preparations derived from the IgG portion of anti-IgM<sup>Slo</sup> (Fig. 6) or normal rabbit IgG (data not shown) did not suppress in vitro release of IgM RF by the patient's MNL.

Although characterization studies did not reveal detectable contaminating undigested IgG in the F(ab')<sub>2</sub> antiidiotypic preparations, additional experiments were undertaken in which the F(ab')<sub>2</sub> fraction of antiidiotypic

type directed against the patient's circulating paraprotein was passed over a protein A-Sepharose CL-4B column before addition to the patient's MNL. Insignificant change in inhibitory activity of the preparation was observed, further confirming the lack of IgG contamination.

*F(ab')<sub>2</sub> antiidiotypic directly suppresses B cell elaboration of idiotype.* To determine whether in vitro suppression of idiotype release by F(ab')<sub>2</sub> antiidiotypic required T cells, peripheral blood MNL from the patient were separated into T cell- and B cell-enriched fractions. As shown in Table II, preincubation of the patient's B cells with F(ab')<sub>2</sub> antiidiotypic suppressed release of IgM RF (Table II, experiment A), whereas

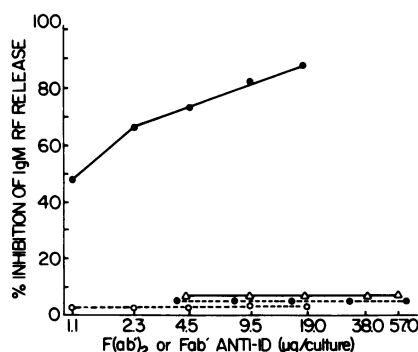


FIGURE 6 Suppression of in vitro IgM RF release by the F(ab')<sub>2</sub> fraction of antiidiotypic antibody. MNL ( $2 \times 10^5$ /culture) obtained from patient Mil were preincubated for 48 h with F(ab')<sub>2</sub> (● — ●) or Fab' (● - - - ●) fragments derived from antiidiotypic serum prepared against the patient's IgM RF paraprotein or with F(ab')<sub>2</sub> (○) or Fab' (Δ) fragments derived from antiidiotypic serum prepared against an unrelated IgM RF paraprotein. The cells were then washed and incubated in medium alone for 5 d at 37°C in 5% CO<sub>2</sub> in air atmosphere. At the termination of culture, cell supernatants were harvested by centrifugation and assayed for IgM RF by solid-phase RIA. Results are expressed as percentage of inhibition of IgM RF release in comparison with MNL preincubated in medium alone.

TABLE II  
F(ab')<sub>2</sub> Antiidiotypic Antibody Directly Suppresses B Cell Release of IgM RF

Reagent	IgM RF	Inhibition
	ng/culture	%
Experiment A*		
None	372	—
F(ab') <sub>2</sub> anti-IgM <sup>Mil</sup> (10 µg/culture)	61	84
F(ab') <sub>2</sub> anti-IgM <sup>Slo</sup> (10 µg/culture)	348	6
Experiment B†		
None	336	—
F(ab') <sub>2</sub> anti-IgM <sup>Mil</sup> (10 µg/culture)	336	—
F(ab') <sub>2</sub> anti-IgM <sup>Slo</sup> (10 µg/culture)	480	—

\* Isolated B cells ( $1 \times 10^5$ ) preincubated in medium alone or F(ab')<sub>2</sub> antiidiotypic for 48 h, washed three times, and then incubated in medium alone for 5 d.

† Isolated T cells ( $2 \times 10^5$ ) preincubated in medium alone or F(ab')<sub>2</sub> antiidiotypic for 48 h, washed three times, and then cultured with B cells ( $1 \times 10^5$ ) for 5 d. T cells alone released 4.2 ng IgM RF.

identical treatment of the patient's T cells did not suppress subsequent B cell synthesis of IgM RF when the fractions were co-cultured (Table II, experiment B).

**Antiidiotypic antibody suppresses synthesis of idiotype.** The suppression of idiotype expression by antiidiotypic antibody could have resulted from either inhibition of synthesis and/or secretion of idiotype. To distinguish these possibilities, we examined intracellular levels of IgM and IgM RF after treatment of the patient's MNL with F(ab')<sub>2</sub> antiidiotypic antibody. As depicted in Table III, decreased extracellular and intracellular IgM and IgM RF was observed after treatment with a suppressive dose of antiidiotypic antibody.

## DISCUSSION

In agreement with animal studies (5-8), the capacity of antiidiotypic antibody to suppress human idiotype expression has been demonstrated both in vitro (32-34) and in vivo (35). Despite the acknowledged efficacy of antiidiotypic antibody in modulating idiotype expression in man, little information has been forthcoming concerning the mechanisms involved. In this study, we analyzed the in vitro influence of well-characterized antiidiotypic reagents on idiotype expression by monoclonal peripheral blood B cells obtained from a patient with B cell lymphoma. Unlike previous human studies analyzing the influence of antiidiotypic reagents (34), addition of polyclonal activators (e.g., pokeweed mitogen) was not required in this study, since the patient's B cells spontaneously elaborated IgM that consisted entirely of monoclonal IgM RF bearing the same idiotype as the patient's circulating IgM RF paraprotein. Our studies were, therefore, directed at determining cellular and molecular requirements for suppression of idiotype expression by the patient's B cells.

The data indicate that both IgG and F(ab')<sub>2</sub> preparations of antiidiotype were capable of inhibiting release of idiotype in vitro (Figs. 5 and 6), whereas IgG

or F(ab')<sub>2</sub> fractions prepared from normal rabbit serum or an unrelated antiidiotypic serum (raised against another IgM $\kappa$  RF) were inactive. The activity of the antiidiotypic antibody was specific in that in vitro IgM and IgM RF production by MNL obtained from unrelated healthy adults was not affected. Furthermore, anti- $\mu$  antibody was <1% as potent as antiidiotypic antibody in suppressing idiotype expression. Although the IgG and F(ab')<sub>2</sub> antiidiotype preparations exhibited similar antiidiotype activity as assessed in a sensitive binding-inhibition assay (Fig. 3), the IgG fraction was approximately five times more effective than the F(ab')<sub>2</sub> preparation in suppressing in vitro IgM RF release by the patient's blood MNL. In view of previous reports that F(ab')<sub>2</sub> antiidiotypic antibody was ineffective in inhibiting murine (16, 17) and human (33) idiotype production, we were concerned that the activity of the F(ab')<sub>2</sub> preparation was related to contaminating IgG. Three lines of evidence argue against this possibility: (a) Analysis of F(ab')<sub>2</sub> preparations by polyacrylamide gel electrophoresis in SDS buffer and Sephadex G-100 column chromatography did not reveal evidence of contaminating intact IgG, (b) ultracentrifugal analysis of the F(ab')<sub>2</sub> fragments revealed the presence of a homogeneous preparation (mol wt = 97,400) without detectable contamination by intact IgG, and (c) passage of F(ab')<sub>2</sub> preparations over a protein A-Sepharose column did not remove significant antiidiotypic activity or alter the capacity of these preparations to suppress in vitro idiotype release. In contrast, passage of the parent IgG antiidiotype preparation over the same column completely removed both activities. Taken together, these data clearly indicate that the bivalent F(ab')<sub>2</sub> fragment of antiidiotypic antibody is sufficient to suppress release of idiotype. Our results, however, also support the view that the Fc portion of antiidiotypic antibody does contribute to suppression of idiotype, perhaps through Fc receptors (17), since the IgG fraction was clearly more potent than F(ab')<sub>2</sub> preparations.

In view of the capacity of the bivalent F(ab')<sub>2</sub> fragment to suppress in vitro release of idiotype, it was of interest to also examine the activity of monovalent Fab' fragments. It was important first to demonstrate that these preparations retained antiidiotypic activity, as in the binding-inhibition assay (Fig. 3), which established that Fab' was approximately one-third as active as the IgG and F(ab')<sub>2</sub> antiidiotype preparations in this regard. Despite demonstrable antiidiotypic activity, Fab' preparations failed to suppress in vitro idiotype release (Fig. 6). Taken together, our data therefore suggest that cross-linking of cell surface idiotype is sufficient to prevent idiotype release.

We next turned our attention to the cellular requirements for in vitro suppression by antiidiotypic

TABLE III  
Antiidiotypic Antibody Suppresses In Vitro  
Synthesis of Idiotype

Reagent	Extracellular		Intracellular	
	IgM	IgM RF	IgM	IgM RF
	ng/culture			
None	138	148	3.6	3.9
F(ab') <sub>2</sub> anti-IgM $\kappa$ <sup>Mil</sup> (19 $\mu$ g/culture)	<3	<1	<1.1	1.4
F(ab') <sub>2</sub> Normal rabbit (19 $\mu$ g/culture)	129	132	3.8	3.5



antibody. This issue has received limited attention in man. Animal studies have indicated that antiidiotypic antibody may modulate idiotypic expression either through T cells (2, 36, 37) or directly at the B cell level (38, 39). Our studies clearly indicate that antiidiotypic antibody is capable of suppressing idiotypic expression at the B cell level without a requirement for intermediary T cells (Table II). These results are in agreement with recent studies of Geha and Communale (40) demonstrating that antiidiotype is capable of specifically inhibiting idiotypic expression at the B cell level.

Information concerning the mechanisms by which antiidiotypic antibody suppresses expression of idiotypic is limited. In this regard, the recent studies of Bonagura et al. (34) indicating that antiidiotype is capable of suppressing the appearance of idiotypic-positive plasma cells in a pokeweed mitogen driven system are of considerable interest. The observation that  $F(ab)_2$  antiidiotype suppressed both extracellular and intracellular IgM and IgM RF in our patient suggest that antiidiotype suppresses synthesis, not secretion, of idiotypic. Although we cannot completely exclude a lytic effect of the antiidiotypic preparations on idiotypic-positive cells, it should be stressed that these studies were all performed in heat-inactivated fetal calf serum, which argues against a role for complement. Moreover,  $F(ab)_2$  antiidiotype preparations did not decrease spontaneous uptake of labeled thymidine by the patient's cells (data not presented).

The suppression of idiotypic synthesis by antiidiotypic antibody observed in our studies may be related to the phenomenon of effector cell blockade initially described by Schrader and Nossal (41). In these experiments multivalent antigens were observed to inhibit specific antibody secretion by antibody forming cells. More recent in vitro studies suggest that effector cell blockade is not dependent upon Fc receptors and is reversible (42). The precise mechanisms by which multivalent ligands suppress secretion of antibody is unclear, although the elegant study of Hannestad and Gaudernack (43) has suggested that secreted antibody may be arrested in transit through the cell membrane by surface-bound ligands. Such a mechanism, however, seems unlikely to account completely for the suppression observed in our studies, since decreased synthesis of idiotypic was clearly demonstrable after treatment with antiidiotype.

In summary, we have demonstrated that antiidiotypic antibody is capable of suppressing spontaneous synthesis of idiotypic at the B cell level without mediation of T cells. The absolute molecular requirement for the bivalent idiotypic binding fragment ( $F(ab)_2$ ) in order to achieve suppression suggests that cross-linking of B cell surface idiotypic is sufficient to inactivate subsequent synthesis of idiotypic. Further studies are

called for to delineate the molecular events occurring subsequent to interaction of cell surface idiotypic with antiidiotype that culminate in inactivation of idiotypic synthesis.

## ACKNOWLEDGMENTS

The authors appreciate the expert technical assistance of Cindy Moore and Maria Frangakis and the help of Joyce Cook in preparing the manuscript. The authors are also grateful to Dr. Alan Solomon for the kind gift of serum from patient Slo. We also wish to acknowledge the many helpful suggestions of Dr. Max Cooper and the critical comments of Drs. Jiri Mestecky and John Kearney concerning the manuscript.

This work was supported by research grants AM 03555 and AM 32073 from the National Institutes of Health.

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