Antiguanosine Antibodies in Murine and Human Lupus Have the Internal Image of G-Binding Proteins

KEITH K. COLBURN, ANDREW L. WONG, RICHARD H. WEISBART, and LORA M. GREEN

ABSTRACT. ***Objective.*** To examine the binding specificities of serum IgG antibodies of mouse and human origin directed against guanosine. The immunodominance of guanosine compared with the other nucleosides was established in the MRL/lpr murine model of systemic lupus erythematosus (SLE). Serum antiguanosine autoantibodies in human lupus correlate with nephritis and polyserositis in acute disease as well as in exacerbations of disease symptoms.

Methods. Antiguanosine autoantibodies obtained from humans with SLE were compared to a murine monoclonal antiguanosine antibody, 4H2. The fine specificity of the antiguanosine-binding site was determined by methylation of specific positions on the guanosine molecule and using defined analogs in competitive ELISA.

Results. Competitive inhibition assays revealed that serum antiguanosine antibodies bind across the 1 and 7 positions of the guanosine molecule ($p < 0.01$) and that an oxygen is necessary at position 6 in the molecule. 4H2 exhibited the same binding specificity for guanosine as human polyclonal antiguanosine antibodies, showing a conserved epitope across species. When the fine specificity was compared with known epitopes, the antiguanosine antibodies were found to have the internal image of a G-binding protein, identical to that of the Ha-ras oncogene product p21.

Conclusion. The finding that antiguanosine autoantibodies vary directly with specific features of SLE, especially nephritis and polyserositis, suggests that they may contribute to the pathology of SLE. Our findings that antiguanosine antibodies have G-binding protein active site homology support the possibility that this species of antibody might interfere with cell signal transduction. (J Rheumatol 2003;30:993–7)

Key Indexing Terms:
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A variety of autoantibodies are produced in patients with systemic lupus erythematosus (SLE), including antibodies (Ab) to polynucleotides and to individual nucleosides[1-3]. Specific antibodies to the nucleosides have been described[4,5]. Stollar and Borel demonstrated the immunodominance of naturally occurring autoantibodies to guanosine in murine models of SLE[6]. Additionally, Stollar induced antibodies to nucleic acids and found that antiguanosine antibodies were the predominant anti-nucleotide antibody species produced[7]. Subsequent studies of the antigenicity of nucleosides in humans with SLE revealed a high degree of specificity of serum antiguanosine antibodies (anti-G antibodies) that correlated with specific features of lupus when compared with other autoimmune diseases, thus supporting the immunodominant role for guanosine[3]. The specificity of anti-nucleoside antibodies has been reported[8-10]. Munns, *et al* described specific antigenic domains or epitopes on the nucleosides cytosine and guanosine[11,12]. Weisbart, *et al* described the fine specificity of IgG anti-G antibodies, comparing them to polyspecific IgM anti-G antibodies of mouse and human origin[10]. Additional studies showed that serum anti-G antibodies in SLE patients were significantly reduced by treatment with corticosteroids and plasmapheresis[13,14]. Moreover, serum anti-G antibodies correlated with disease activity in patients with procainamide-induced SLE, especially the arthritis and polyserositis features of the disease[9]. Recently, we reported that serum anti-G antibodies in 86 patients with SLE correlated more closely with SLE disease activity than did anti-double stranded (ds) DNA antibodies, especially with regard to nephritis and polyserositis[15]. In that study we also showed that patients followed longitudinally (3–7 years) had serum

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anti-G antibody concentrations that varied directly with disease activity as measured by the SLE Disease Activity Index (SLEDAI)[16].

We examined whether anti-G antibodies from humans with SLE and MRL/lpr mice showed similar immune reactivity to guanosine and whether the murine MRL/lpr monoclonal anti-G antibody (anti-G Mab) 4H2 shares the same guanosine epitope as anti-G autoantibodies isolated from humans with SLE.

MATERIALS AND METHODS

Patients. As part of a Veterans Administration approved study, sera from 86 patients (80 women, 6 men) with SLE were screened for anti-G autoantibodies. The average age of all the patients was 28.1 years (range 14–77 yrs). Seven patients were taking azathioprine 100–150 mg/day, 46 prednisone (or prednisone equivalent) 5–120 mg/day, and 6 cyclophosphamide 75–100 mg/day or intravenous cyclophosphamide pulses. Sera from 48 of these patients were selected who had active SLE as determined by the Revised American College of Rheumatolgy (ACR) criteria[17] and the SLEDAI[16] and had relatively high levels of circulating anti-G autoantibodies[15].

Fine specificity of anti-G antibodies. Competitive inhibition assay. To determine the location or epitopes on the guanosine molecule that antibodies to guanosine recognized we utilized a competitive ELISA. Guanosine was conjugated to human serum albumin and bound to microtiter plates as described[18,19]. The plates were treated with 10% fetal calf serum (FCS) to occupy any uncoated sites. Separately, guanosine was modified by methylation at the 1, 2, 3, or 7 position in the double ring structure. The modified guanosine was incubated with patient sera overnight at 4°C to ensure sufficient time for the excess modified guanosine to bind to the sera anti-G antibodies. Then the supernatant was transferred to the guanosine-coated plates. At the end of the incubation period, the plates were washed and alkaline phosphatase-secondary goat anti-human IgG added to all wells in the plates. Controls included human serum albumin and phosphate buffered saline (PBS) to blank the wells. The excess secondary antibody was removed by washing and the substrate added, and the plates were read at 405 nm wavelength. The level of IgG bound to the plates was inversely related to the ability of the modified guanosine to compete by preoccupying the active sites of the anti-G antibodies and thus prevent them from binding to the native guanosine coated plates.

Cross-reactivity assays. Determining cross-reactivity of anti-G antibodies with DNA, other nucleosides, and guanosine analogs were performed by competitive inhibition ELISA like those described above. The following reagents were used in excess to compete with anti-G for guanosine: calf thymus native (ds) DNA, ss-DNA from denatured calf thymus, cytidine, thymidine, adenosine, and guanosine analogs, hypoxanthine, acyclovir, guanine, guanadine, and cyclic GMP.

Monoclonal antibody 4H2 preparation and characterization. Anti-G antibodies are spontaneously produced in MRL/lpr and NZB-NZW hybrid female mice. Anti-G Mab 4H2 was produced from MRL/lpr spleen cells fused with the FOX-NY4 myeloma cell line, a spontaneous mutant of NS-1, and selected with IXAAT medium as described[20]. The culture supernatants were screened with guanosine conjugated to human serum albumin by ELISA. Hybridoma cells secreting anti-G antibodies were cloned by limiting dilution and injected into pristane-primed BALB/c mice for ascites production. Characterization of the specificity anti-G Mab 4H2 was performed using a standard ELISA[19]. The results indicated that 4H2 bound to guanosine, ss-DNA, and ds-DNA attached to poly-L-lysine[21,22] but not to the other free nucleosides (cytidine, thymidine, and adenosine).

Statistical analysis. The differences between the various methylated guanosine preparations and the guanosine controls in the blocking experiments were calculated from the resulting corrected optical densities (OD) using geometric (log) means by one-way analysis of variance with 99% confidence levels (CI) determined *post hoc* by the Fisher PLSD test and the Scheffe F-test (Stat View, Brain Power Inc., Calabasas, CA, USA, for repeated measured ANOVA transformed data).

RESULTS

Specificity of patient sera for free nucleosides. Sera from the 48 patients used in these studies were tested individually by competitive ELISA to determine the level of cross-reactivity of their serum immunoglobulin to the free nucleoside bases of DNA. Figure 1 shows the data for the combined values ± SEM. When guanosine was incubated with the serum samples prior to testing in the guanosine-coated plates, it was able to prevent > 75% of the maximum signal, whereas adenosine blocked 15%, thymidine 5%, and cytidine 3% of the signal. Thus there was relatively little cross-reactivity of the patient sera for the other nucleosides. We have reported that anti-G antibodies bind to ss-DNA and to a lesser extent ds-DNA[15].

Fine specificity of human antiguanosine autoantibodies. Contribution of positions 1, 2, 3, and 7 in the guanosine molecule to antiguanosine binding. To determine which positions on the guanosine molecule were important to the specificity, we modified guanosine by adding methyl groups individually to positions 1, 2, 3, and 7 in separate reactions. The human anti-G antibody-containing sera were tested by competitive ELISA to determine the contribution of each of the respective sites to binding on the guanosine molecule. In all assays, unmodified guanosine was used as a control and it competitively inhibited 75–90% of the anti-G from

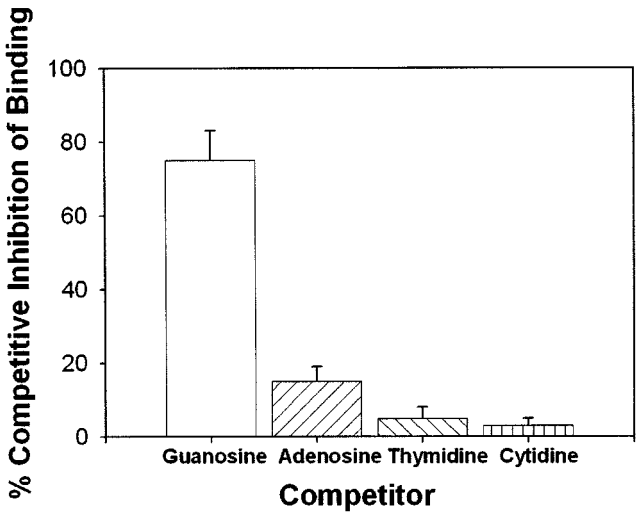

Figure 1. Competitive ELISA to determine the affinity of antiguanosine for free nucleosides. The free nucleoside guanosine is shown to successfully compete (78 ± 8%) for antiguanosine, thereby preventing it from binding to the guanosine-coated plate. The other nucleosides of DNA (adenosine, thymidine, and cytidine) do not bind to the active sites on antiguanosine and therefore do not prevent the antiguanosine from binding. The results shown are the average of values from 48 patients (± SEM). The difference between the abilities of guanosine and the other nucleotides to compete for antiguanosine was significant ($p < 0.0001$).

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binding to the guanosine coating the plates (Figure 2). The results indicated that methylating position 1 reduced competitive binding by 70% and methylating position 7 reduced competitive binding by 60%. The differences between the control and the modified 1 and 7 positions were statistically significant ($p < 0.01$). Methylating the nitrogen molecules at positions 2 and 3 in the guanosine molecule gave results similar to unmodified guanosine, in that they occupied the antibody binding sites, preventing anti-G from binding to the guanosine coated plates [N2 (84%) and 3 (96%)]. These findings indicated that the binding of anti-G to guanosine involved epitopes that were across the 1 and 7 positions of the guanosine double ring structure.

Contribution of position 6 in the guanosine molecule to antiguanosine binding. The findings that positions 1 and 7 were important to the binding suggested that the intervening oxygen at the 6 position may also contribute to the binding specificity of anti-G for guanosine. Sera from 15 patients with the highest concentrations of serum anti-G antibodies were further tested to determine whether position 6 was important in the recognition of the guanosine molecule by anti-G. Cyclic GMP, guanine, acyclovir, and hypoxanthine are analogs of guanosine that have oxygen or a hydroxyl group (OH) at position 6. These analogs were used in competitive ELISA to assess the contribution of an oxygen molecule at position 6 in the basic double ring structure. Additionally, adenosine was used in these experiments because it differs from guanosine by the presence of an amine (NH2) at position 6. The results from 3 duplicate experiments (± SEM) are shown in Figure 3. cGMP and acyclovir were equivalent to unmodified guanosine in their ability to bind to anti-G. Hypoxanthine was about 30% as effective as guanosine at competing for the antibody active site, and adenosine was about 20% effective. The findings indicated that the presence of an oxygen or a OH radical at 6 position on the double-ringed structure enhanced whereas an amino group inhibited binding of anti-G antibodies, suggesting that an oxygen molecule at the 6 position may be necessary for binding anti-G antibodies to guanosine.

Fine specificity of murine antiguanosine antibody 4H2. To determine the specificity of anti-G antibodies obtained from the mouse model of SLE, we repeated the human experiments described above using the murine anti-G Mab, 4H2. Figure 4 shows the compiled data from 5 triplicate experiments (± SEM) that show 4H2 binding to guanosine involves positions 1 and 7 similar to results with polyclonal human sera. Binding of anti-G Abs to guanosine was completely blocked by methylation of guanosine at the 1 and 7 positions. Modifying position 2 at the nitrogen was not effective. The Mab 4H2 was also competitively inhibited by guanosine analogs with an oxygen or OH radical at position 6 on the double ring structure but not by adenosine. The findings indicate that there was cross species conservation of binding properties of anti-G Abs to guanosine.

DISCUSSION

Our results provide evidence through epitope mapping that anti-G Abs found in sera from humans with SLE bind the identical locations on the guanosine molecule as the Mab anti-G, 4H2, isolated from the murine model of SLE. Our findings suggest the importance of highly specific Abs in the pathogenesis of certain features of SLE and lead one to consider the possible pathogenic importance of highly specific binding patterns and epitopes associated with the

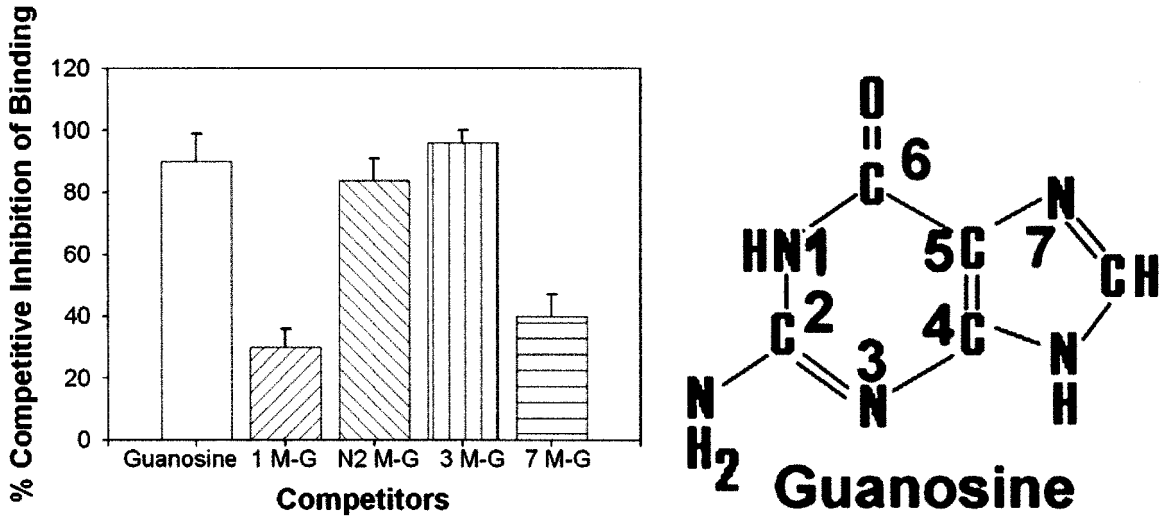

Figure 2. Contribution of positions 1, 2, 3, and 7 in the guanosine molecule to antiguanosine binding. Methylation of positions 1 or 7 on the double ring structure of guanosine prevented the modified molecule from binding to antiguanosine. Comparisons of unmodified guanosine to the modified molecule (at positions 1 or 7) were significant ($p < 0.01$). As the modification places the methyl group farther from the 1 and 7 positions, the less effect it has on guanosine-antiguanosine interaction. Methylation of the nitrogen at position 2 or 3 in guanosine was nearly equivalent to unmodified guanosine ($p > 0.5$).

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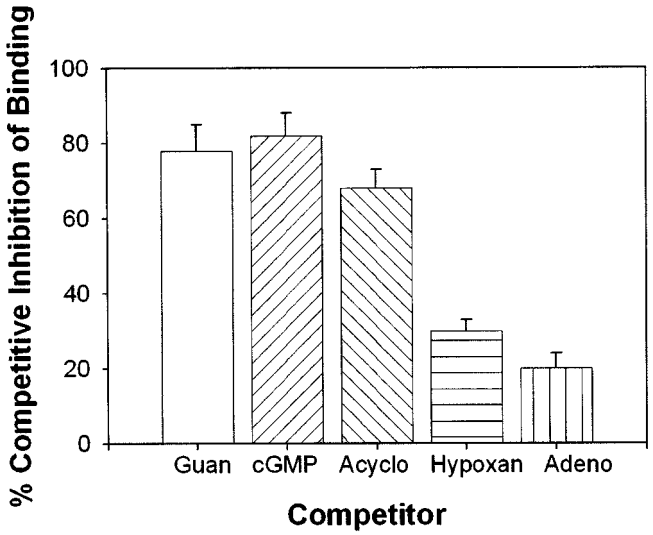

Figure 3. Contribution of position 6 in the guanosine molecule to antiguanosine binding. Analogs of guanosine with oxygen or a hydroxyl group at position 6 on the guanine double ring structure were assessed for their ability to interfere with antiguanosine binding to unmodified guanosine by competitive ELISA. Cyclic guanosine monophosphate (cGMP) and acyclovir (acyclo) were equivalent to native guanosine with respect to their ability to block binding of antiguanosine to guanosine ($p > 0.5$). Hypoxanthine was less competitive (30%) and adenosine, which has an amine group at position 6, was able to block only 20% of antiguanosine-guanosine binding ($p < 0.001$).

nucleosides. Our study confirms previous work by Munns, *et al* showing that the Ab binding site (epitope) on the guanosine molecule for IgG anti-G Abs is across positions 1 and 7 of the guanine double ring[11]. We showed that oxygen or an OH radical at position 6 on the guanine molecule was essential for anti-G antibody binding. We also showed that these exact binding specifications on guanosine are conserved in the Mab 4H2 of MRL/lpr origin, an animal model for lupus nephritis. Signal-transducing guanine-binding proteins (G binding proteins) exert their function by cycling between an inactive GDP-containing and an active GTP-containing conformation described by Pai, *et al* to be structurally the same as the Ha-ras oncogene product p21[23]. G-binding proteins activated by the GDP to GTP reaction then activate adenyl cyclase, which in turn activates production of the second messenger, c-AMP. Our study shows that anti-G antibodies bind to GDP and GTP at the same epitope as G-binding proteins, indicating that anti-G antibodies have the same internal image as G-binding proteins. Whether anti-G antibodies interfere with cell signal transduction is of great interest to our laboratory and is currently under investigation.

Highly specific anti-G antibodies provide a marker for SLE disease activity in nephritis and polyserositis in humans[15]. The IgG anti-G mouse Mab 4H2 binds to the

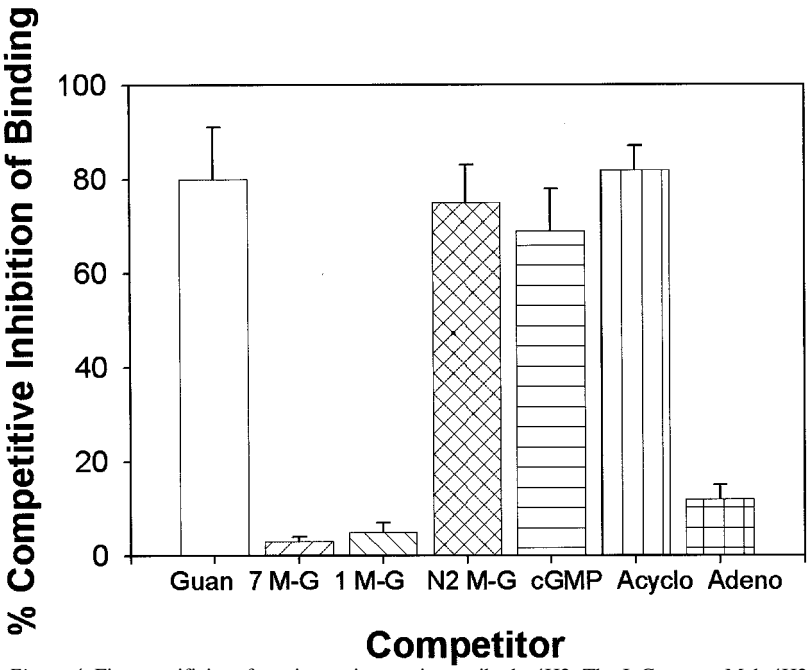

Figure 4. Fine specificity of murine antiguanosine antibody 4H2. The IgG mouse Mab 4H2 was subjected to the same competitors as used for the human polyclonal antiguanosine. When tested with native guanosine there was roughly 80% blocking of 4H2 (antiguanosine) binding to the guanosine coated plates. Guanosine modified at positions 1 and 7 was unable to compete for binding. This is similar to the human IgG anti-G antibodies, where methylation at positions 1 and 7 on the guanine double ring blocked binding. As seen with the human antibody, methylating position 2 was not effective. The oxygen or hydroxyl containing analogs of guanosine (cGMP and acyclovir) were able to successfully compete and thereby block binding, whereas adenosine was not competitive.

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same eptiope on the guanine double ring structure as anti-G antibodies found naturally occurring in human SLE. The Mab 4H2 provides a tool to further our understanding of the mechanisms of antigen-antibody binding at a molecular level and should help delineate the potential pathogenicity of these antibodies that are associated with several features of active SLE.

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