Vol. 136, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 14, 1986 Pages 145-150

NATURALLY OCCURRING MASKED ANTIBODIES IN MURINE SERA RECOGNIZE A COMPONENT OF THE MITOTIC SPINDLE APPARATUS

Theodore W. Munns, M. Kathryn Liszewski, and Sandra K. Freeman

Washington University School of Medicine,
Division of Rheumatology, Box 8045, St. Louis, MO 63110

Received February 14, 1986

SUMMARY Antibody activities previously masked in autoimmune MRL and normal Balb/c mice were expressed by briefly subjecting their sera to acidic (pH 2.0) or alkaline (pH 12.0) environments. An enzyme-linked immunosorbent assay (ELISA) revealed that these pH-expressable immunoglobulins reacted with specific nucleotide-BSA antigens (primarily 5'-AMP, -GMP, -TMP) but not with single (ss)- or double (ds)-stranded DNAs or with unconjugated BSA. ELISA analysis of pH-expressed antibodies purified via GMP-BSA/Sepharose indicated that they bound not only to the homologous hapten (GMP) but to AMP and TMP as well, i.e., anti-pAGT antibodies. Further, indirect immunofluorescent assays (IIF) with fixed HEp-2 cells demonstrated that purified anti-pAGT antibodies recognized an epitope within the mitotic spindle apparatus. These results document the existence of a previously undefined masked antibody population in murine sera with specificities directed toward certain nucleotides and a component of the mitotic spindle apparatus. Last, these anti-pAGT (or anti-mitotic spindle) antibodies are not restricted to murine systems inasmuch as they have been detected in every human serum (>100 samples) examined thus far.

The existence of anti-DNA antibodies in autoimmune diseases in general and systemic lupus erythematosus (SLE) in particular has been documented (1-3). Such populations appear to be heterogeneous in view of the multitude of reported specificities, e.g., base, nucleoside, and/or nucleotide components, oligonucleotide sequences, sugar-phosphate units, ss- and dsDNA conformations, and B- and Z-configurations (2,3). Generally most specificity studies have utilized either unbound serum antibodies or monoclonal preparations with only limited attention focused upon similar characterization of bound populations. While anti-DNA antibody:DNA complexes have been detected (4-6), the specificity of these masked immunoglobulins remains undefined. To obtain this information we briefly exposed both autoimmune (MRL) and normal (Balb/c) serum to pH environments that would dissociate suspected complexes. Upon neutralization, pH-treated

0006-291X/86 $1.50

serum was subjected to ELISA analysis for detection of previously masked anti-DNA antibodies. While this procedure was unable to detect masked anti-DNA antibodies in murine sera, it was successful in establishing the existence of a unique population of masked antibodies that reacted with nucleotides (ELISA) and a component of the mitotic spindle apparatus (IIF).

METHODS AND MATERIALS Sera were obtained from 18 to 20 wk old autoimmune MRL (7) and normal Balb/c mice, heat inactivated, and stored at -20°C. Nucleotides (5'-AMP, -GMP, etc, including 5'-PO_4-ribothymine) were purchased from Sigma and conjugated to BSA according to established procedures (8) as was the conjugation of nucleotide-BSA antigens to Sepharose. DNA reagents included ssDNA (Sigma), dsDNA (Miles), and DNA-agarose (Bethesda Research). Fixed HEp-2 cells (human epithelial) were obtained from Kallestad and indirect immunofluorescent assays (IIF) performed according to the manufacturer's instructions. Other immunochemical reagents employed in ELISA or IIF were obtained from Sigma.

For removal of unbound anti-nucleotide and -DNA antibodies, both MRL and Balb/c serum were diluted 10-fold in phosphate-buffered saline (PBS; 10 mM PO_4, 150 mM NaCl, pH 7.4) containing 0.05% Tween-20 and 2 mg/ml BSA (i.e., PBS-TB). Diluted serum (1.0 ml) was incubated with DNA-agarose (0.1 ml) for 2 hr at 24°C prior to centrifugation. ELISA analysis of the supernatant (designated preadsorbed serum) revealed the effectiveness of this procedure to remove both unbound anti-nucleotide and -DNA antibodies (see Table 1). To dissociate suspected anti-DNA antibody:DNA complexes, preadsorbed serum was titrated to pHs of 2.0 and 12.0 with 1.0 N HCl and KOH, respectively. After a 15 min incubation period (24°C) in these pH environments, preadsorbed serum was neutralized (pH 7.2 ± 0.2) and immediately processed for ELISA and IIF testing. Procedural details regarding both ELISA (9,10) and IIF (11,12) have been described.

For affinity purification of pH-expressed anti-nucleotide antibodies, preadsorbed and pH-treated serum (see above) was incubated with 0.1 volume of GMP-BSA/Sepharose for 1 hr at 24°C. The adsorbent was washed successively with PBS-BT, 0.1 M $NaHCO_3$ (pH 8.5), and 0.5 M NaCl containing 10 mM PO_4 (pH 7.4) via repeated centrifugation-resuspension. Antibodies retained by GMP-BSA/Sepharose were eluted wth 1.0 ml aliquots of acidified BSA (2 mg/ml BSA, pH 2.0), neutralized, and diluted with PBS-BT prior to ELISA and IIF assessments. Identical studies were conducted with DNA agarose and Sepharose adsorbents containing BSA and 5'-AMP (see Table 2).

RESULTS AND DISCUSSION Recently we have documented the utility of ELISA for evaluating the specificity of anti-nucleic acid antibodies (9,10). However, since these sera were not subjected to conditions which promote dissociation of immune complexes, results pertaining to specificity were restricted to unbound antibody populations. Accordingly, we initiated studies to determine if pH treatments of sera would result in the expression of masked anti-DNA antibodies. The results of these investigations (Table 1) revealed the following information. First, the binding of antibody to nucleotide-BSA

Table 1: pH-Dependent Expression of Anti-Nucleotide Antibodies Derived from the Serum of MRL and Balb/c Mice[a]

Serum (Dilution) Treatment[b]	pH	Nucleotide-BSA Antigens						DNA Antigens	
		BSA	pA	pG	pC	pU	pT	ssDNA	dsDNA
		Adsorbance (405 nm)[d]							
MRL-pool[e](1:2500)									
Untreated	7.3	ND[c]	.18	.38	ND	.05	.16	.70	.29
Preadsorbed	7.3	ND	ND	ND	ND	ND	ND	ND	ND
Preadsorbed	12.0[f]	ND	.52	.75	.24	.26	.60	ND	ND
Preadsorbed	2.0[f]	ND	.40	.64	.19	.16	.53	ND	ND
Balb/c-pool[e](1:250)									
Untreated	7.2	ND	.06	.20	ND	.08	.12	.08	ND
Preadsorbed	7.3	ND	ND	ND	ND	ND	ND	ND	ND
Preadsorbed	12.0	ND	.36	.46	.20	.24	.42	ND	ND
Preadsorbed	2.0	ND	.31	.40	.18	.20	.37	ND	ND

[a]Aliquots of serum (either untreated or DNA-agarose adsorbed) were incubated in either acidic (pH 2.0), alkaline (pH 12.0), or neutral (pH 7.3) environments for 15 min prior to neutralization, dilution (as indicated), and ELISA analysis of antibody binding to a spectrum of immobilized antigens. [b]Treatment: sera employed in these experiments were used without manipulation (untreated) or adsorbed with DNA-agarose (preadsorbed) prior to pH adjustment and ELISA assessment. [c]ND: adsorbance not detected, or less than 0.04 A_{405} units. [d]Adsorbance: values listed above represent the mean values derived from three experiments. Variation of individual values was less than 10% of the reported mean when the latter was greater than 0.15 A_{405} units. [e]MRL- and Balb/c-pool: while these data were derived from pooled sera (10 mice) numerous individual sera yielded similar results. [f]12.0 and 2.0: these pHs represent optimum expression of masked antibody activities.

antigens was specific for the nucleotide component since immobilized BSA was unreactive. Second, unbound anti-nucleotide and anti-DNA antibodies were effectively removed from serum by DNA-agarose, i.e., compare A405 values of untreated serum versus preadsorbed-(pH 7.3) serum. Third, exposure of preadsorbed serum to either acid (pH 2.0) or alkaline (pH 12.0) environments resulted in the expression of previously masked antibodies that reacted with AMP, GMP, TMP (primary) and CMP, UMP (secondary) but not with ss- or dsDNA antigens. Further, the quantity of pH-expressable antibodies exceeded by 2- to 5-fold those representative of unbound populations in untreated sera. Fourth, pH-treatment of normal sera (Balb/c) revealed a similar enhancement in the types of antibody expressed, yet the quantity of masked antibodies in Balb/c serum was less than 10% of those present in autoimmune mice. Quantitative differences were based upon the extent each serum had to be diluted to achieve similar absorbance values (see Table 1).

Table 2. Isolation of pH-Expressed Antibodies By Various Adsorbents as Assessed via ELISA [a]

Adsorbents	Nucleotide-BSA Antigens						DNA Antigens	
	BSA	pA	pG	pC	pU	pT	ssDNA	dsDNA
	Adsorbance (405 nm)							
DNA-agarose	ND[b]	ND	ND	ND	ND	ND	ND	ND
BSA/Seph	ND	ND	ND	ND	ND	ND	ND	ND
AMP-BSA/Seph	ND	.47	.52	.28	.22	.50	ND	ND
GMP-BSA/Seph	ND	.54	.59	.28	.26	.52	ND	ND

[a]Affinity purification procedures for the isolation of pH-expressed antibodies from MRL preadsorbed serum are described in METHODS. Antibodies retained by these adsorbents were acid-eluted, neutralized, diluted (1:1000), and subjected to ELISA analysis. [b]ND: absorbance not detected or less than 0.04 A405 units. Based upon 3 adsorption studies with GMP-BSA/Sepharose, the recovery of pH-expressed immunoglobulins binding to GMP-BSA (ELISA) was 30 to 40%.

Because of their marked specificity towards mononucleotides, we attempted to purify pH-expressable immunoglobulins by their adsorption to nucleotide-BSA/Sepharose adsorbents. Antibodies retained by the various adsorbents employed were acid-eluted, neutralized and reevaluated via ELISA. Our findings (Table 2) indicate the successful isolation of pH-expressed antibodies by both AMP- and GMP-BSA/Sepharose but not by BSA/Sepharose or DNA-agarose. The observation that antibodies purified with either AMP- or GMP-BSA/Sepharose reacted equally well with AMP, GMP, and TMP (ELISA) suggest a monospecific population (i.e., anti-pAGT) that may recognize an oligonucleotide sequence. Such sequence specificity would account for the inability of anti-pAGT antibodies to bind with ssDNA since this antigen conceivably lacks the requisite sequence or is minimally represented in this regard.

While the ELISA provided sufficient information for detecting (Table 1) and isolating (Table 2) anti-pAGT, the identity of the cellular antigen(s) recognized by these immunoglobulins remained unresolved. Accordingly, purified anti-pAGT antibodies together with MRL serum were incubated with fixed HEp-2 cells. As assessed by immunofluorescent staining (Figure 1) unfractionated MRL serum yielded fluorescence throughout the entire cell nucleus (panel A) particularly in those cells undergoing mitotic division.

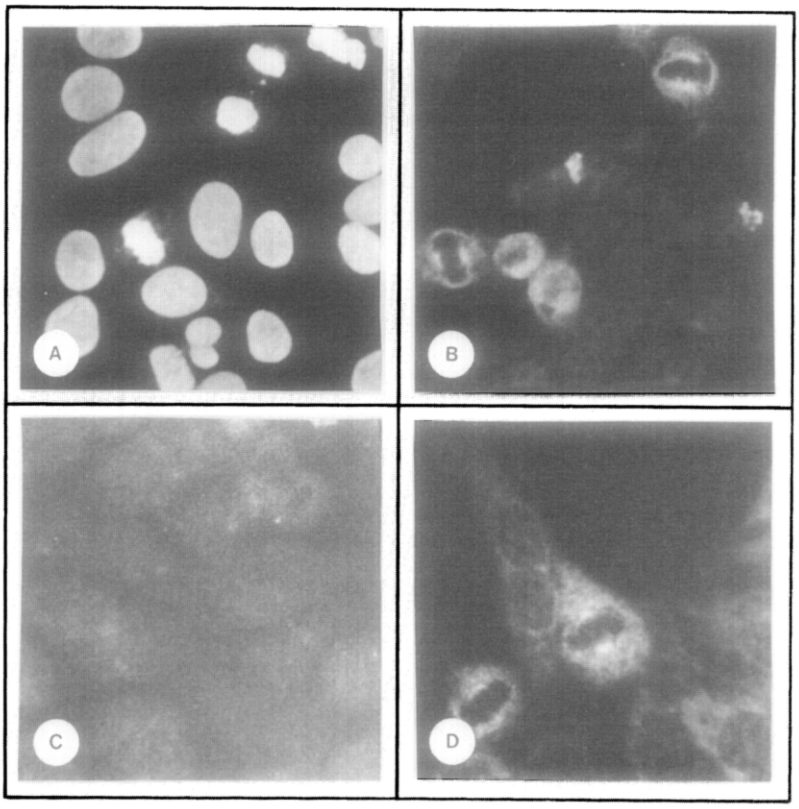

FIGURE 1: Immunofluorescence of HEp-2 cells after incubation with untreated MRL serum (1:100 dilution; panel A), with affinity purified MRL anti-pAGT antibodies (1:50 dilution; panel B), with untreated Balb/c serum (1:20 dilution; panel C), and with alkaline-treated Balb/c serum (1:20 dilution; panel D). Each preparation was diluted (as indicated) with PBS-BT and incubated with cells for 20 min at 24°C. Glass slides containing these fixed cells were washed in PBS (2X, 5 min each) and reincubated with FITC-goat anti-mouse IgG for 20 min prior to additional washings (as above) and microscopic examination. Resulting fluorescent patterns were photographed through a Leitz (Ortholux) fluorescent microscope. Photomicrographs represent a magnification of 400X.

This homogeneous pattern is typical in SLE and indicative of multiple autoantibodies including those reacting with nucleic acids (1). In marked contrast, purified anti-pAGT antibodies reacted predominantly with spindle fibers of mitotic cells (panel B). Similar IIF assessment of untreated Balb/c serum yielded negligible fluorescence throughout the entire cell (panel C). However, an identical aliquot preexposed to either an acidic or alkaline environment resulted in the expression of masked anti-pAGT antibodies (Table 1) that reacted exclusively with mitotic spindle fibers (panel D). Results identical to the above have been obtained not only with

pooled and individual murine sera but with human sera as well. Of particular interest in our human studies was the finding that levels of pH-expressable anti-pAGT (or anti-spindle fiber) antibodies in healthy subjects are comparable to those present in active SLE patients.

Collectively, these results document the existence of a previously undescribed population of masked antibodies in both murine and human sera that possess specificities directed towards nucleotides (AMP, GMP, TMP) and react with some component of the mitotic spindle apparatus. Unlike other anti-spindle fiber antibodies that are present infrequently (<0.1%) in autoimmune sera (11,12), the antibodies described herein exist in masked form, react with specific nucleotides, and are present in all human and murine sera (i.e., ca. 150 samples) examined thus far.

ACKNOWLEDGEMENTS The authors gratefully acknowledge Drs. John Atkinson and Jeffrey Kaine for their advice and critical discussions of these investigations. This research was supported by grant CA 27801 awarded by the National Institutes of Health and by the Lottie Caroline Hardy Trust for Arthritis Research.

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