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Immunology

Monoclonal IgM antibody exhibiting high-affinity binding and cryoglobulin properties

(antifluorescein/class switch/autoimmunity/affinity maturation)

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ABSTRACT **A monoclonal IgM antibody (18-2-3) derived from cell fusion of (NZB × NZW) F_1 splenocytes following secondary immunization with fluorescein-conjugated keyhole limpet hemocyanin was shown to exhibit high intrinsic binding affinity and cryoinsolubility. Affinity-purified preparations were determined to be IgM by immunochemical, electrophoretic, and chromatographic analyses. An intrinsic association constant (K_a) of 2.9×10^{10} M^{-1} (at 2°C) was measured by first-order dissociation-rate analysis. Antibody solubility at low concentration (≈50 μg/ml) was shown, by absorption spectroscopy, to be temperature dependent between 4°C and 32°C. Insolubility at low temperature (4°C) was reversible in the presence of homologous fluorescyl hapten, indicative of active site involvement in the mechanism of cryoglobulin–18-2-3 complex formation. Characteristics of clone 18-2-3 are discussed in terms of (*i*) its potential use as a model for examining the mechanism of cryoprecipitation and (*ii*) the proposed relationship between affinity maturation and the IgM to IgG class switch.**

A functional characteristic of the humoral immune response to repeated challenge with certain antigens is a temporal increase in the average intrinsic association constant (K_a) of specific antibody (1, 2). Quantitative measurement of the relative affinities of IgG and IgM antibodies elicited to T-dependent hapten-carrier systems have indicated that the process of affinity maturation is largely restricted to the IgG class (3). In what may be a related finding, amino acid and DNA sequence analyses of immunoglobulin heavy chain variable regions from phosphorylcholine binding antibodies have shown extensive IgG heavy chain variable region diversity relative to their IgM counterparts (4, 5). Thus, it has been suggested that displacement of IgM by predominantly IgG-producing B cells during antigen-driven clonal expansion may temporally limit the extent to which somatic diversification mechanisms act on IgM germ-line variable gene segments (6). However, the molecular and cellular bases involved in the coordination of Ig class switching and variable region somatic variation remain undefined.

To approach various questions concerning the structural and functional correlates of antibody affinity maturation and diversity, the fluorescein hapten system has been used in the examination of both heterogeneous and homogeneous antibodies. Fluorescein–protein conjugates elicit an immune response in rabbits (7, 8) and BALB/c mice (9) that exhibits significant time-dependent maturation of antibody affinity. Nine monoclonal IgG antibodies derived from a secondary BALB/c response collectively exhibited an extensive range of affinities ($K_a = 10^6$ to 10^{10} M^{-1}) and diverse binding mechanisms for the fluorescyl ligand (10, 11). By contrast, in the antifluorescyl response of New Zealand Black (NZB) mice, a strain that develops autoantibodies cytotoxic for T suppressor cells at an early age (12, 13), affinity maturation appears to occur more rapidly within the primary response (9).

In the present study, characterization of a monoclonal IgM antifluorescyl antibody derived from (NZB × NZW) F_1 hyperimmune splenocytes is described. IgM clone 18-2-3 is atypical, relative to previously characterized antihapten monoclonal IgM antibodies, with respect to two functional properties: (*i*) it exhibits a cryoglobulin-like temperature-dependent insolubility that is inhibited on fluorescyl ligand binding and (*ii*) it has an intrinsic association constant for fluorescein of 2.9×10^{10} M^{-1}.

MATERIAL AND METHODS

Immunizations. Fluorescein isothiocyanate (isomer I) was covalently conjugated to keyhole limpet hemocyanin (KLH) as described (14). Male (NZB × NZW) F_1 mice obtained from our breeding colonies were immunized intraperitoneally with 200 μg of the conjugate emulsified in complete Freund's adjuvant (day 0). Splenic lymphocytes were harvested for cell fusion on the fourth day following secondary immunization (day 21).

Cell Lines and Fusions. Hybridomas were produced by polyethylene glycol fusion of the Sp 2/0-Ag 14 myeloma cell line with immune splenocytes, followed by hypoxanthine/aminopterin/thymidine selection, and cloning in 0.2% agar (15). Antifluorescein-secreting hybrids were detected by a liquid-phase radioimmunoassay as described (10) and propagated in pristane-primed BALB/c mice to induce ascites fluid. Monoclonal antifluorescyl antibodies (4-4-20, 4-6-9, 4-6-10, and 16-19-1) from BALB/c-derived hybridoma cell lines have been described in detail elsewhere (10, 11).

Antibody Purification. Lipoproteins were removed from BALB/c ascites fluids by sodium dextran sulfate precipitation and the gamma globulin fraction was obtained by precipitation with 50% saturated ammonium sulfate. Antifluorescyl antibodies were affinity purified from the gamma globulin fraction by immunoadsorption on fluorescein-Sepharose 4B and subsequent elution of specific antibody in the presence of 0.1 M disodium fluorescein (8). Unbound dianionic fluorescein was removed by anion exchange chromatography on Dowex 1-X8 and eluted antibodies were monitored for absorbance at 278 and 500 nm to determine the relative concentrations of protein and bound fluorescein, respectively.

Electrophoresis. $NaDodSO_4$/polyacrylamide gel electrophoresis was carried out using a 12% acrylamide gel in the discontinuous $NaDodSO_4$ buffer system of Laemmli (16) as described by Watt and Voss (17). Samples containing 2% $NaDodSO_4$

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Abbreviation: KLH, keyhole limpet hemocyanin.

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were reduced with 0.1 M dithiothreitol, alkylated with 0.2 M iodoacetamide, and electrophoresed at 15 mA for ≈4 hr. Resolved heavy and light chain polypeptides were stained with Coomassie blue and apparent molecular weights were determined by interpolation from relative mobility curves constructed with protein standards run in parallel.

Fluorescence-Quenching Measurements. Fluorescein is significantly quenched (>90%) when bound by heterogeneous rabbit antifluorescein (18). Maximum fluorescence-quenching values (Q_{max}) for monoclonal antifluorescyl antibodies were determined by titration of 1 ml of 0.1 μM fluorescein with increasing volumes of gamma globulin fractions (11). Samples were excited at 480 nm and fluorescence was monitored through a 500-nm cutoff filter (Corning 3-69) at 535 nm in an Aminco-Bowman spectrophotofluorometer equipped with a Heath recorder (model EVW-20A) and Forma Scientific controlled-temperature bath and circulator.

Previous studies have shown that involvement of hydrogen bonding in fluorescein binding and quenching can be evaluated by determining the relative quenching in ^2H_2O and in H_2O (19, 20). The fluorescence of antibody-bound fluorescein in ^2H_2O and H_2O was compared by adding 50 μl of affinity-purified antibody (7.2 μM active sites) containing bound fluorescyl ligand (5.4 μM) to a mixture of 920 μl of ^2H_2O or H_2O and 30 μl of 2 M Tris·HCl (pH 8.0) (11). Samples were incubated for 3 hr at room temperature and the fluorescence was monitored as described above.

Dissociation Rates and Derived Association Constants. Dissociation rates of fluorescein bound to antifluorescyl antibodies were determined as described (19). One hundred microliters of affinity-purified antibody (1.4 μM sites) containing 1 μM fluorescein in 50 mM KPO_4 (pH 8.0) was placed in a cuvette at 2°C and then 1 ml of 1 μM 5-aminofluorescein was added. Fluorescence intensity was monitored over time under the conditions described above. Because 5-aminofluorescein has a comparatively low quantum yield (0.003 compared with 0.92 for fluorescein), yet binds antifluorescyl antibodies with the same affinity as fluorescein (21), an increase in fluorescence due to dissociation of previously quenched bound ligand is observed. Fluorescence dissociation data were analyzed and plotted by first-order kinetics using Eq. 1 (19):

$$\ln \frac{(FU)_m - (FU)_t}{(FU)_m - (FU)_0} = -kt, \quad [1]$$

where $(FU)_0$ is the initial fluorescence, $(FU)_t$ is the fluorescence observed at time t, and $(FU)_m$ is the maximum fluorescence observed on complete dissociation of bound fluorescein. K_a values were determined from association rates (k_1) and dissociation rates (k_2):

$$Ab + Fl \underset{k_2}{\overset{k_1}{\rightleftharpoons}} AbFl \quad [2]$$

$$K_a = \frac{k_1}{k_2}, \quad [3]$$

where Ab is antibody concentration, $k_1 = 5 \times 10^6\ M^{-1}s^{-1}$ at pH 8.0 for most antifluorescyl antibodies (11), and k_2 was measured as described above.

Cryoglobulin Studies. The effects of temperature and ligand binding on monoclonal antibody solubility were examined by absorption spectroscopy. Measurements were made with a double-beam Cary (model 219) recording spectrophotometer equipped with a Frigidflow bath circulator (New Brunswick). The globulin fractions of monoclonal antibody preparations were initially clarified by centrifugation at ambient temperature and the optical densities at 278 nm were adjusted to ≈1.0 in 50 mM KPO_4 (pH 8.0). Samples were equilibrated at 32°C and the absorbance at 320 nm (22) was recorded as a function of temperature with decrements of 1.5°C/min. The effect of fluorescyl ligand on cryoprecipitation was measured by titration of 2 ml of gamma globulin ($A_{278} \approx 1.1$) with 0.1 mM disodium fluorescein at 4°C, and the transmitted light at 320 nm was recorded as a function of ligand concentration.

RESULTS

Identification of Immunoglobulin Class. Nine antifluorescyl monoclonal antibodies derived from a BALB/c secondary response to the fluorescein-KLH conjugate (including clones 4-4-20, 4-6-9, 4-6-10, and 16-19-1 used in the present study) have been previously shown to be IgG (10). Clone 18-2-3, derived from a (NZB × NZW) F_1 secondary antifluorescein response, was IgM by several criteria. First, the apparent molecular weights of resolved heavy and light chains from affinity-purified antibodies were determined by $NaDodSO_4$/polyacrylamide gel electrophoresis (Fig. 1). Reduced and alkylated preparations of clone 18-2-3 consisted of heavy and light chains with apparent molecular weights 70,100 and 28,900, respectively. Spreading of the heavy chain pattern can be attributed to the duration of reduction and alkylation rather than to copurified contaminants. Second, the heavy chain isotype was verified and light chain class was determined by double diffusion against appropriate anti-mouse reagents (data not shown) to be μ and κ, respectively. Finally, clone 18-2-3 did not bind to protein A-Sepharose CL-4B, which is specific for murine IgG isotypes γ1, γ2a, γ2b, and γ3 (23).

Ligand Binding Studies. Significant amounts of bound fluorescein remained in affinity-purified preparations of clone 18-2-3 after removal of free ligand by Dowex 1-X8 anion exchange chromatography. The r value (mol of fluorescein bound per mol of antibody monomer), calculated based on A_{278} and A_{500} measurements (18), indicated that ≈75% of total antibody combining sites were ligand bound. The affinity of 18-2-3 for fluorescein and various fluorescent properties of the bound state were quantitated and compared with those of a BALB/c-derived IgG antifluorescyl monoclonal antibody (4-4-20) displaying the highest K_a ($1.7 \times 10^{10}\ M^{-1}$) from a panel of IgG hybridoma proteins (10). Values for the intrinsic association constant were determined by analyses of first-order dissociation rates of

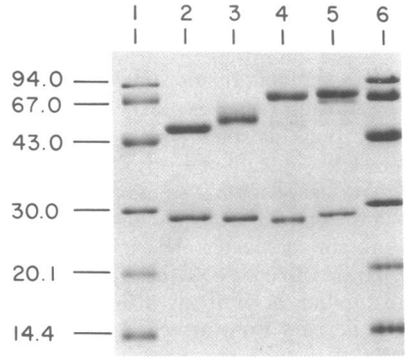

FIG. 1. $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of monoclonal antifluorescyl 18-2-3 antibodies (lane 5). Affinity-purified samples were reduced and alkylated prior to electrophoresis on a 12% slab. Molecular weight standards (lanes 1 and 6), IgG antifluorescyl clones 4-6-9 and 6-19-1 (lanes 2 and 3), and IgM antinucleotide clone 11-7-6 (lane 4) were run as markers. Numbers on the left represent $M_r \times 10^{-3}$.

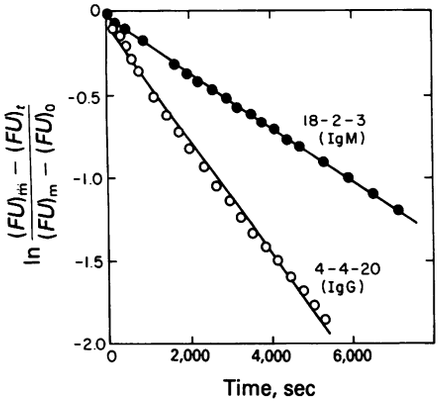

FIG. 2. Dissociation rates of fluorescein bound to purified monoclonal antifluorescyl preparations. One milliliter of 1 μM 5-aminofluorescein was added to 100 μl of antibody (1.4 μM sites) containing ≈75% bound fluorescein. Samples were excited at 480 nm and the increase in fluorescence was monitored at 535 nm as a function of time.

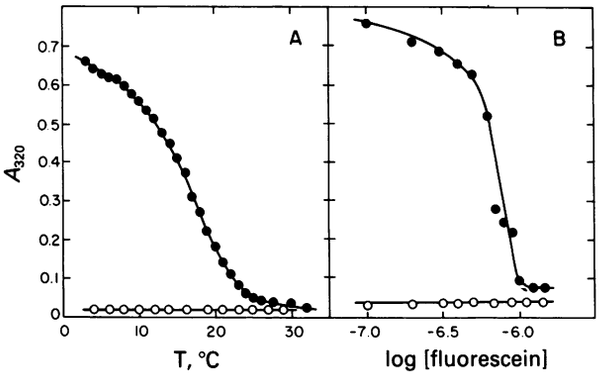

FIG. 3. Cryoglobulin properties of clone 18-2-3. (*A*) Effect of temperature on 18-2-3 (●) cryoinsolubility. Absorbance at 320 nm of gamma globulin fractions ($A_{278} \approx 1.0$) was monitored between 4°C and 32°C. (*B*) Effect of homologous hapten on 18-2-3 (●) cryoinsolubility. Two milliliters of gamma globulin was titrated with 0.1 mM fluorescein at 4°C and A_{320} was measured after each 2-μl aliquot. For both experiments, antifluorescyl clone 4-6-10 gamma globulin (○) was used as a control.

bound ligand (Fig. 2). Homogeneity of both preparations was shown by linear dissociation rate plots (correlation coefficient = 0.99). The time required for bound ligand to reach e^{-1} (lifetime τ) was calculated by linear regression analysis of the slopes of the curves (Table 1). The intrinsic association constant derived from the dissociation lifetime for 18-2-3 at 2°C was 2.9×10^{10} M^{-1}. Scatchard analysis of equilibrium measurements (fluorescence quenching) made at 24°C (data not shown) confirmed the relatively high binding affinity of 18-2-3 ($K_a > 10^8$ M^{-1}), although precise measurements were complicated by the large fraction of ligand-bound antibody in affinity-purified preparations and cryoprecipitation in gamma globulin fractions (see below). Comparison of Q_{max} values at ligand saturation and the absorption maxima (λ_{max}) of bound relative to free fluorescein (λ_{max} = 493 nm) indicated that clones 18-2-3 and 4-4-20 quenched ligand fluorescence on binding (>95%) and the resultant bathochromic shifts in absorption λ_{max} were of comparable magnitudes (10–12 nm). However, ^2H_2O perturbation of ligand-bound preparations resulted in significant fluorescence enhancement of 4-4-20-bound fluorophore only (187.9%). Together, these data (summarized in Table 1) suggested a marked difference in the degree to which hydrogen bonding, relative to hydrophobic interactions, contributes to the high binding affinity of each clone (11).

Cryoglobulin Properties. During the course of these studies, it was observed that 18-2-3 gamma globulin fractions became markedly turbid on storage at 4°C. It was also noted that such turbidity was readily reversed by warming to 37°C. Because this phenomenon was not observed with any of nine BALB/c-derived monoclonal antifluorescyl antibodies (10), it was of interest to examine clone 18-2-3 as a potential model for the structural basis of such cryoglobulin properties. Using absorbance at 320 nm as an index of turbidity (22), we measured the degree of cryoinsolubility at different temperatures at low specific antibody concentration (≈50 μg/ml). As shown in Fig. 3*A*, 18-2-3 solubility was markedly temperature sensitive between 4°C and 32°C. As indicated above, BALB/c-derived IgG monoclonal antibodies (4-6-10; Fig. 3*A*, and other data not shown) did not exhibit cryoinsolubility at comparable concentrations of gamma globulin ($A_{278} \approx 1.0$). Hence, it is possible that the unique cryoglobulin properties of 18-2-3 may be associated with its origin [(NZB × NZW) F_1], Ig heavy chain class, or the mechanism that generated a high-affinity binding site.

To evaluate the role of the active site in 18-2-3 cryoprecipitation, a fluorescein ligand inhibition experiment was carried out. As shown in Fig. 3*B*, complete reversal of cryoinsolubility at low temperature (4°C) could be achieved by binding of homologous fluorescyl hapten. The latter property was consistent with our inability to detect cryoprecipitation in affinity-purified 18-2-3 preparations, which retained significant amounts of bound ligand (≈75% saturation) after anion exchange chromatography. Although these results show that the active site of 18-2-3 is involved in cold-induced insolubility, it remains to be determined whether 18-2-3 requires an additional component for cryoprecipitation or self-aggregation is involved. However, $NaDodSO_4$/polyacrylamide gel electrophoretic patterns of gamma globulin cryoprecipitates isolated by centrifugation at 4°C (data not shown) appeared similar to affinity-purified and fluorescein-eluted 18-2-3 (Fig. 1), suggesting a single component IgM cryoglobulin.

Table 1. Comparison of properties of BALB/c- and F_1-derived high-affinity monoclonal antifluorescyl antibodies

	Clone	
	4-4-20	18-2-3
Murine strain	BALB/c	NZB × NZW F_1
Isotype (heavy, light)	γ2, κ	μ, κ
Dissociation lifetime,* sec	1,683	5,783
K_a,† M^{-1}	1.7×10^{10}	2.9×10^{10}
Q_{max},‡ %	96.4	99.4
Absorption maximum,§ nm	505	503
^2H_2O enhancement,¶ %	187.9 ± 19	−3.8 ± 2.8

* Lifetime τ calculated from slopes of dissociation rate plots (Fig. 2).
† $K_a = k_1\tau$, calculated for 18-2-3 using an average k_1 of 5×10^6 M^{-1} sec^{-1} (11).
‡ Bound fluorescein relative to free fluorescein.
§ Value for bound fluorescein.
¶ Fluorescence of bound fluorescein in ^2H_2O relative to fluorescence in H_2O.

DISCUSSION

A unique property of clone 18-2-3 is its temperature-dependent solubility. Absorption spectroscopy studies show that the solubility of hybridoma protein 18-2-3 decreases on temperature reduction (Fig. 3*A*) and cryoprecipitation at low temperature is inhibited by homologous fluorescyl ligand (Fig. 3*B*), implying that the mechanism of cryoglobulin 18-2-3 precipitation involves the active site. Considering the exceptional ab-

sorption and fluorescence properties of the fluorescyl ligand (18), such a hybridoma protein may be uniquely suitable for analysis of various cryoglobulin physiocochemical properties, in particular the previously proposed active-site conformational changes that occur on temperature reduction (24).

Previous affinity maturation studies examining heterogeneous IgG and IgM responses to T-dependent immunogen systems have indicated that only IgG antibodies exhibit significant time-dependent increases in affinity (for review, see ref. 3). Accordingly, nine antifluorescyl monoclonal antibodies (K_a = 10^6–10^{10} M^{-1}) derived from BALB/c mice after secondary immunization (10) have been shown to be of the IgG class (IgG1 or IgG2). Using identical immunization, cell fusion, and hybridoma-screening protocols, we obtained a monoclonal IgM antibody from fusion products of hyperimmune (NZB × NZW) F_1 splenocytes that possessed an exceptionally high binding affinity ($K_a = 2.9 \times 10^{10}$ M^{-1}) for fluorescyl ligand. Like the NZB parent, (NZB × NZW) F_1 progeny spontaneously develop thymocytotoxic autoantibodies that can potentially compromise T-cell function at an early age (12, 13). In addition, immunoglobulin expression in BALB/c and NZB mice differs significantly by several criteria: (*i*) the kinetics and extent of affinity maturation within the NZB primary response to fluorescyl haptenic antigen resembles the BALB/c antifluorescyl secondary response with respect to these maturation parameters (9); (*ii*) comparative analyses of class distribution, antigen-binding properties, and variable region amino acid sequences of BALB/c and NZB myeloma proteins indicate that these randomly derived pools are structurally and functionally distinct (25); and (*iii*) NZB mice spontaneously express antibodies to erythrocyte and lymphocyte self-antigens (26) and exhibit a high incidence of cryoglobulin formation, particularly of the IgM class (27). Thus, isolation of a unique high affinity IgM antibody from a (NZB × NZW) F_1 mouse may be associated with such abnormalities. Other monoclonal antihapten (nucleotide-KLH) antibodies derived from F_1 mice in our laboratory have also shown a high proportion (60%) of μ heavy chains (unpublished data).

It has been suggested (4–6, 28) that antibodies containing μ heavy chains express variable region germ-line genes that may undergo somatic mutation in association with the class switch to γ heavy chains. Such variant immunoglobulins may then exhibit higher binding affinities due to altered variable region residues. Alternative explanations for such observations are (*i*) the process of IgM to IgG switching causes somatic mutational events, (*ii*) somatic mutations of germ-line genes are temporally associated with the class switch, or (*iii*) expression of somatically mutated genes leads to a switch from IgM to IgG (perhaps due to T-cell recognition of altered residues). Indirect evidence that somatic mutation may occur before class switching in the phosphorylcholine-KLH response has recently been reported (29). If affinity maturation requires somatic mutation, as has been suggested (5), the existence of a high-affinity IgM antibody necessarily eliminates alternative *i*.

The ability to derive an antigen-induced cryoglobulin from (NZB × NZW) F_1 hybrids may be related to the numerous immunological abnormalities noted above. Interestingly, other studies have shown that persistent challenges with streptococcal antigen can lead to cryoglobulin formation (30). Similar to clone 18-2-3, such antigen-induced cryoglobulins appear to be components of hyperimmune responses. Hence, the etiology of abnormal solubility properties may be associated with certain events that occur during maturation of the response. As indicated above, it has been suggested that the process of affinity maturation may involve somatic mutational events that result in the production of an antibody molecule with altered binding properties (5). A similar mechanism (i.e., mutation at framework positions) has been proposed as the molecular basis for the temperature-dependent insolubility of two human cryoglobulins (31). Thus, it will be of importance to determine whether the high-affinity binding and cryoglobulin properties exhibited by 18-2-3 are structurally related.

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