Anti-metatype Antibodies Stabilize the Fluorescein Single-chain Antibody 4–4–20 Complex against Dissociation by Hydrostatic Pressure*

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Hydrostatic pressure was used to promote dissociation of fluorescein (Fl) from single-chain antibody 4–4–20 (SCA 4–4–20). Fl fluorescence intensity was quenched by 97% upon binding to SCA 4–4–20. Increasing pressure to 2.4 kbar enhanced Fl fluorescence from the remaining 3% to 14–17%. The capacity of anti-metatype antibodies (anti-Met), which specifically recognize liganded anti-Fl antibodies, to protect against pressure-induced Fl dissociation was tested. Both polyclonal and monoclonal anti-Met antibodies protected against Fl dissociation, reducing the fluorescence intensity at 2.4 kbar from 14–17% to 6–8%. Additive effects of anti-Met antibodies in protection against pressure-induced Fl dissociation were suggested by the fact that a 2-fold molar excess polyclonal anti-Met reagent promoted additional protection relative to an equimolar amount. On the other hand, combination of different monoclonal anti-Met antibodies did not promote additive protection, suggesting recognition of overlapping metatopes by these monoclonals. The complex formed by SCA 4–4–20 and Fl analog HPF was more sensitive to pressure-induced Fl dissociation than the unliganded Fl–mAb complex. Addition of both polyclonal and monoclonal anti-Met antibodies reduced the Fl fluorescence recovery at 2.4 kbar from 75% to 40–55%. In order to directly study binding of anti-Met antibodies to mAb 4–4–20, monoclonal anti-Met antibody 3A5–1 was labeled with 2-dimethylaminonaphthalene-5-sulfonyl chloride (2,5-Dns-C1) and Dns fluorescence anisotropy measured. Unliganded mAb 4–4–20 did not bind to 2,5-Dns-3A5–1 as indicated by the absence of measurable changes in Dns fluorescence anisotropy upon increasing mAb concentration. Addition of mAb 4–4–20 bound to Fl produced a sigmoidal increase in Dns anisotropy, compatible with association of the primary immune complex and 3A5–1. An affinity constant, \(K_{D,5}\), of 1.5 × 10^–8 M and a cooperativity coefficient (\(n\)) of 3.1 were calculated for formation of the Fl-mAb 4–4–20 complex. The HPF-mAb 4–4–20 complex was also recognized by 2,5-Dns-3A5–1 but with lower affinity, indicating that the monoclonal anti-Met 3A5–1 distinguished between mAb 4–4–20 liganded to different haptons.

Anti-metatype antibodies (anti-Met) are immunoglobulins that specifically recognize antibodies in the liganded state but lack specificity for either the ligand alone or the unliganded antibody (Voss et al., 1988, 1989). Metatopes, i.e., determinants specifically recognized by anti-Met antibodies, have been proposed to originate from conformational changes induced by ligand binding. Such conformational changes are associated with the immunoglobulin variable regions, since anti-Met antibodies recognize ligand-bound single-chain antibody derivatives comprised of the heavy and light chain variable region domains of the original monoclonal antibody (Weidner and Voss, 1991). Bound anti-Met antibodies delay the dissociation rate of the Fl and anti-Fl antibody complex (Voss et al., 1988; Weidner and Voss, 1991; Weidner et al., 1992). It has been shown that the mechanism responsible for such delay in dissociation rate involves a decrease in conformational fluctuations undergone by the Fl-anti-Fl antibody complex (Weidner et al., 1992). The fact that anti-Met antibodies delay antigen dissociation in vitro correlates with a recent finding that rabbits elicit an autologous anti-Met response in vivo (Voss et al., 1992) and supports the idea that anti-Met antibodies could play an important role in controlling the physiological clearance of immune complexes from the circulation (Voss, 1993).

As noted above, a systematic characterization of anti-Met antibodies has been performed to date, using the Fl hapten model (Voss et al., 1988; Weidner and Voss, 1991; Weidner et al., 1992; Weidner et al., 1993). Nevertheless, these studies have employed measurements of dissociation rate kinetics to study the effects of anti-Met antibodies on dissociation of Fl from anti-Fl antibodies. In such measurements, Fl dissociation is induced by addition of the nonfluorescent Fl analog fluoresceinamine, which replaces Fl in the anti-Fl antibody binding site (Herron, 1984). This approach permitted studies of the influence of anti-Met antibodies on the affinity of anti-Fl antibodies for Fl. However, direct determination of affinity constants of anti-Met antibodies for the Fl-anti-Fl complex are still lacking.

It has recently been shown that hydrostatic pressure promotes dissociation of Fl from the single-chain antibody 4–4–20 (SCA 4–4–20) (Coelho-Sampaio and Voss, 1993). In the present work, hydrostatic pressure was combined with fluorescence measurements to investigate the ability of anti-Met antibodies to protect against pressure-induced dissociation of the Fl-SCA 4–4–20 complex. In addition to polyclonal anti-Met antibodies, monoclonal anti-Met, 3A5–1, 1A4, PlE11, and 2C3 (Weidner et al., 1993), were also used in this study. We show that both polyclonal and monoclonal anti-Met antibodies protected against pressure-induced dissociation of Fl from SCA 4–4–20. Dissociation of the Fl analog 9-hydroxyphenylfluoruron (HPF) was also examined. In addition, determination of the equilibrium constant involved in dissociation of the ternary complex fluorescein; SCA, single-chain antibody; mAb, monoclonal antibody; HPF, 9-hydroxyphenylfluoruron; \(K_{D,5}\), concentration of free ligand necessary to occupy one-half of the antibody binding sites; 2,5-Dns-C1, 2-dimethylaminonaphthalene-5-sulfonyl chloride.

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1 The abbreviations used are: anti-Met, anti-metatype antibodies; Fl, fluorescein; SCA, single-chain antibody; mAb, monoclonal antibody; HPF, 9-hydroxyphenylfluoruron; \(K_{D,5}\), concentration of free ligand necessary to occupy one-half of the antibody binding sites; 2,5-Dns-C1, 2-dimethylaminonaphthalene-5-sulfonyl chloride.
MATERIALS AND METHODS

Single-chain Antibody 4-4-20—SCA was constructed, expressed, and isolated from Escherichia coli cells as described elsewhere (Bird et al., 1988; Denzlin et al., 1991).

mAb 4-4-20—mAb 4-4-20 was obtained from mouse ascites fluid by affinity purification using FI-Sepharose 4B as previously described (Kranz and Voss, 1981; Reinitz and Voss, 1984).

Affinity-labeled mAb 4-4-20-Sepharose—Affinity labeling of mAb 4-4-20 was carried out in 0.1 M borate buffer (pH 8.5) by incubating 1:1 (moles of active sites) of mAb 4-4-20 with fluorescein isothiocyanate (Sigma) for 3 h at room temperature. Nonlabeled fluorescein isothiocyanate was removed by extensive dialysis in phosphate buffer, pH 8.0. In the last dialysis change, the buffer was replaced by coupling buffer (0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3). Affinity-labeled mAb 4-4-20 was incubated with CNBr-Sepharose 4B (Sigma) in a rotator for 3 h at room temperature. After incubation, the Sepharose was washed in coupling buffer and the supernatant monitored at A₂₇₈ to determine coupling (moles of active sites) of mAb 4-4-20 with fluorescein isothiocyanate (Sigma) for 3 h at room temperature. Nonlabeled fluorescein isothiocyanate was removed by extensive dialysis in phosphate buffer, pH 8.0.

Polyclonal Anti-Met Antibodies—Xenogenic polyvalent anti-Met antibodies were produced in rabbit by multiple immunizations with affinity-labeled mAb 4-4-20 emulsified in Freund's complete adjuvant (Difco Laboratories) and the γ globulin fraction isolated as described by Weidner and Voss (1991). Further purification of the anti-Met reagent was obtained using an affinity-labeled mAb 4-4-20-Sepharose column.

Monoclonal Anti-Met Antibodies—Hamster monoclonal anti-Met antibodies were obtained and purified from tissue culture medium as described previously (Weidner et al., 1993).

Normal Rabbit IgG—Normal IgG fraction was obtained from nonimmune rabbit serum by removal of lipoproteins (dextran sulfate and CaCl₂ precipitation) followed by successive precipitations with 30% and 50% saturated ammonium sulfate. The γ globulin-enriched precipitate was dissolved in 0.1 M phosphate buffer, pH 8.0, in a volume equivalent to the original serum volume and dialyzed extensively. The γ globulin fraction was applied to a molecular sieve column (Ultrogel AcA 34, LKB) 80 cm x 2 cm equilibrated in 0.1 M phosphate buffer, pH 8.0. The pure IgG fraction was identified based on comparison with molecular weight markers.

Analysis of SCA 4-4-20 Binding to HPF—Binding of SCA 4-4-20 was followed by measuring changes in HPF fluorescence emission spectra. The mAb was labeled by coupling fluorescein isothiocyanate (200 μM in carbonate buffer, pH 9.5, was added to the Sepharose matrix and placed on a rotator at 4 °C for 24 h to mask remaining chemically active groups. The protein-Sepharose matrix was washed sequentially with coupling buffer, 0.1 M acetate ± 0.5 M NaCl, pH 4.0, and 50 mM phosphate buffer, pH 8.0.

Steady-state fluorescence spectra and polarization were measured using an ISS (Champaign, IL) GREG-PC spectrofluorometer. The reported FI fluorescence intensities correspond to the integrated area of the emission spectra at each pressure calculated as I(λ) = da, where λ is the emission wavelength (in nanometers) and I(λ) is the fluorescence intensity at a given wavelength. FI and HPF fluorescence were expressed in terms of percentage of the remaining fluorescence. Binding and pressure application, which 100% is the intensity of the free fluorophores in solution. In FI fluorescence measurements, excitation wavelength was fixed at 485 nm, and emission was scanned between 500 and 600 nm. When fluorescence anisotropy of 2,5-Dns-SAS-1 was measured, the excitation and emission wavelengths were 300 and 480 nm, respectively. These wavelengths were chosen to minimize FI fluorescence interference in experiments where FI was present. In addition, appropriate blanks were subtracted during anisotropy measurements in order to compensate for the enhancement in fluorescence intensity due to FI addition. All fluorescence measurements were performed at 25 °C. Data presented in this work correspond to representative experiments, which were performed at least two times, using different antibody preparations.

Hydrostatic Pressure Experiments—Hydrostatic pressure in the range of 1 bar to 2.4 kbar was achieved using the pressure bomb described by Paladini and Weber (1981). Protein samples were prepared in 10 mM Tris-HCl buffer, pH 8.0. Before being placed in the pressure cuvette, FI fluorescence quenching induced by antibody binding was assayed in a standard fluorescence cuvette. Spectra were taken after 2 min of equilibration at each pressure.

RESULTS

Pressure-induced Dissociation of FI from SCA 4-4-20—Fig. 1 shows the effect of hydrostatic pressure on the fluorescence emission spectrum of FI bound to SCA 4-4-20. As previously reported, binding of FI to an anti-FI antibody induces a pronounced decrease in FI fluorescence intensity (Kranz and Voss, 1981; Kranz et al., 1983). In the experiment shown in Fig. 1, only 3% of the initial fluorescence of free FI (dashed line) was observed after binding of the antibody (continuous line). At 2.4 kbar, the quenching of FI fluorescence induced by antibody binding was partially reversed, and 15% of the free FI fluorescence was recovered (Fig. 1, dotted line). The FI hapten dissociated gradually from SCA 4-4-20 in response to stepwise increases in pressure as judged by the observed increase in FI fluorescence (Fig. 1, inset) and by a parallel decrease in FI fluorescence polarization (Coelho-Sampaio and Voss, 1993).

Effects of Polyclonal and Monoclonal Anti-Met Antibodies on Pressure-induced FI Dissociation from SCA 4-4-20—FI dissociation from SCA 4-4-20 was assayed in the absence (Fig. 2) or in the presence (●) of 0.2 μM polyclonal anti-Met antibodies. Addition of the polyclonal reagent promoted an increase in FI fluorescence at atmospheric pressure from 3 to 6% (Fig. 2). This increase was followed by a significant decrease in FI polarization, which changed from 0.28 to 0.21 upon addition of polyclonal anti-Met antibodies (not shown). FI fluorescence increases due to FI dissociation have been shown to be accompanied by a decrease in FI polarization (Coelho-Sampaio and Voss, 1993). Thus, at atmospheric pressure, addition of polyclonal anti-Met antibodies promoted a small release of FI from SCA 4-4-20. In the presence of polyclonal anti-Met antibodies, however, the effect of pressure on FI dissociation was significantly attenuated (Fig. 2, ○). At 2.4 kbar, the percentage increase in FI fluorescence changed from 6% at atmospheric pressure to 8%, whereas an increase of 3 to 14% was observed in the same pressure range in the absence of anti-Met.
Anti-Met Antibodies Protect against Ligand Dissociation

Monoclonal anti-Met antibody 3A5–1 was also tested for protection against pressure-induced Fl dissociation (Fig. 2, Δ). In the presence of 3A5–1, Fl fluorescence reached 6–7% at 2.4 kbar, which corresponded to one-half the fluorescence intensity observed at 2.4 kbar in the absence of anti-Met. It is interesting to note that in contrast to polyclonal anti-Met, 3A5–1 did not promote an increase in Fl fluorescence at atmospheric pressure. Addition of normal rabbit IgG did not affect pressure-induced Fl dissociation, ruling out the possibility that the protective effect of anti-Met antibodies was due to nonspecific stabilization conferred by the presence of immunoglobulins (Fig. 2, inset).

Additivity of Anti-Met Antibodies Effects— Chromatographic studies on the complex involving polyclonal anti-Met antibodies and liganded SCA 4-4-20 indicated that at least 5 anti-Met Fab fragments bind to each Fl-SCA 4-4-20 complex (Weidner et al., 1992). Therefore, we investigated whether addition of different monoclonal anti-Met antibodies would further protect against pressure-induced dissociation. Fig. 3A shows Fl dissociation profiles in the absence of monoclonal anti-Met (○) and in the presence of 0.1 μM 3A5–1 (Δ), 0.1 μM 3A5–1 plus 0.1 μM 1A4 (●), and 0.1 μM 3A5–1 plus 0.1 μM 1A4 plus 0.1 μM 2C3 (▲). As can be seen, the presence of three different monoclonal anti-Met antibodies did not confer further stabilization as compared with one or two monoclonals. Furthermore, addition of a molar excess of the same anti-Met (2:1 and 3:1 of 3A5–1 to Fl-SCA 4-4-20 complex) did not promote additional protection against Fl dissociation as compared with an equimolar amount of 3A5–1 (Fig. 3B).

Additivity was also investigated using polyclonal anti-Met antibodies (Fig. 4). In the presence of 0.2 μM polyclonal anti-Met, i.e. a 2-fold molar excess with respect to the Fl-SCA 4-4-20 complex (□), the protective effect was more pronounced than in the presence of 0.1 μM (●). This observation suggested that in the heterogeneous polyclonal population, different anti-Met antibodies can present additive effects in protecting against pressure-induced ligand dissociation.

Pressure-induced Dissociation of the Fl Analog HPF—It was previously shown that mAb 4-4-20 recognized the Fl analog HPF, although with an affinity 200 times reduced relative to Fl binding (Bedzyk et al., 1992). Fig. 5 shows that SCA 4-4-20 also bound HPF as indicated by the gradual quenching of HPF fluorescence observed upon addition of increasing amounts of SCA 4-4-20. The plot of bound versus free SCA (inset) allowed calculation of an association constant for the complex HPF-SCA of 5.6 x 10^6 M^-1.

Application of pressure promoted dissociation of the HPF-SCA 4-4-20 complex at three different concentrations of SCA (Fig. 6). At 0.7 μM (●), HPF fluorescence increased from 12% at atmospheric pressure to 42% at 2.4 kbar. When SCA concentration was reduced to 0.3 (○) or 0.1 μM (▲), changes in percent fluorescence were 28 to 75% and 67 to 90%, respectively. In all cases, HPF concentration was fixed at 0.02 μM. As expected for a lower affinity complex, HPF-SCA 4-4-20 was more sensitive to pressure dissociation than the high affinity Fl-SCA 4-4-20 complex (see Coelho-Sampaio and Voas, 1993) (Figs. 1–4). The profile of pressure-induced Fl dissociation is also shown in Fig. 6 for comparison (▲).

Effects of Polyclonal and Monoclonal Anti-Met Antibodies on Pressure-induced HPF Dissociation from SCA 4-4-20—Addition of 0.6 μM polyclonal anti-Met antibodies protected against HPF dissociation (Fig. 7). At atmospheric pressure, addition of anti-Met promoted an increase in HPF fluorescence similar to that observed with the Fl-SCA complex in the presence of polyclonal anti-Met antibodies (Figs. 2 and 4). A slight increase in HPF fluorescence was observed when pressure was raised from 1 bar to 2.4 kbar in the presence of polyclonal anti-Met reagent.

Fig. 8 shows the effect of monoclonal anti-Met antibodies on pressure-induced dissociation of HPF. Addition of 0.3 μM 1A4 (●), 0.6 μM 1A4 (□), 0.3 μM 1A4 plus 0.3 μM 3A5–1 (△), or 0.3 μM 1A4 plus 0.3 μM PlE11 (▲) were all able to protect against pressure dissociation, reducing the final HPF percent fluorescence from 75% in the absence of anti-Met (○) to 45–55%. As observed for Fl dissociation, simultaneous addition of the two different anti-Met antibodies did not promote further stabilization against pressure effects on the HPF-SCA 4-4-20 complex.

Binding of Anti-Met Antibody 3A5–1 to mAb 4-4-20—Monoclonal anti-Met antibody 3A5–1 was labeled with 2.5-Dns-Cl and Dns polarization measured in the presence of increasing concentrations of mAb 4-4-20 either liganded or in the nonliganded state (Fig. 9). In the absence of mAb 4-4-20, the anisotropy of 2.5-Dns-3A5–1 was in the range of 0.03. This value is smaller than expected for a 150-kDa protein, probably indicating local mobility of the Dns probe. Addition of liganded mAb 4-4-20 (1:2.5 of Fl to mAb) promoted an increase in fluorescence anisotropy, which followed a sigmoidal profile (Fig. 9, ○). Increase in anisotropy is consistent with an increase in size of the protein particle to which the probe is attached (Lakowicz, 1983). When the Fl analog HPF was used instead of Fl to complex mAb 4-4-20, an enhancement in Dns fluorescence anisotropy was also observed (Fig. 9, ●). However, the binding curve using the HPF-mAb 4-4-20 complex indicated that the HPF-mAb 4-4-20 complex binds to 2.5-Dns-3A5–1 with lower affinity than the Fl-mAb 4-4-20 complex. This result suggested that anti-Met antibody 3A5–1 distinguished between complexes formed between mAb 4-4-20 and either Fl or HPF.

Measurements of Dns anisotropy at concentrations of Fl-mAb 4-4-20 or HPF-mAb 4-4-20 higher than 0.5 μM were offset by the significant interference due to the ligand's fluorescence emission. When unliganded mAb 4-4-20 was tested, no significant change in anisotropy of the anti-Met-Dns conjugate was detected (Fig. 9, △). Upon further increase of mAb 4-4-20 concentration up to 1.2 μM, the anisotropy remained unchanged (not shown). This result indicated that monoclonal anti-Met antibody 3A5–1 did not recognize mAb 4-4-20 in the absence of ligands.

Data corresponding to binding of 2.5-Dns-3A5–1 to Fl-mAb
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FIG. 3. Effect of different monoclonal anti-Met antibodies on F1 dissociation. Panel A, F1 dissociation was measured in the absence (○) or in the presence of the following concentrations of monoclonal anti-Met antibodies: 0.1 μM 3A5-1 (△), 0.1 μM 3A5-1 plus 0.1 μM 1A4 (□), or 0.1 μM 3A5-1 plus 0.1 μM 1A4 plus 0.1 μM 2C3 (▲). Panel B, F1 dissociation was measured in the absence (○) or in the presence of 0.1 (△), 0.2 (▲), or 0.3 μM 3A5-1 (□).

FIG. 4. Effect of polyclonal anti-Met antibodies at different concentrations. Pressure-induced F1 dissociation was measured in the absence (○) or in the presence of 0.1 μM (●) or 0.2 μM polyclonal anti-Met antibodies (□).

4-4-20 were further analyzed in a plot of anisotropy against concentration of free F1-mAb complex (concentration of complex that is not bound to 2,5-Dns-3A5-1). Free complex concentration was calculated based on the degrees of dissociation (α) at each point, which takes into account the anisotropies obtained at the lower plateau (α = 0) and at the higher plateau (α = 1). As shown in the inset of Fig. 9, the plot of anisotropy against free complex (instead of total concentration of complex) was sigmoidal and was therefore analyzed using a binding model in which cooperativity is present. A binding curve was fit to experimental data using the following equation:

\[ r = r_i + \left[ r_f Ab^n/(K_{0.5} + Ab^n) \right] \]  

(Eq. 1)

where \( r \) is the anisotropy measured at each antibody concentration, \( r_i \) is the anisotropy at the lower plateau, \( r_f \) is the anisotropy at the higher plateau, \( Ab \) is concentration of free F1-mAb complex, \( n \) is the Hill coefficient and \( K_{0.5} \) is the concentration of free complex at which \( \alpha = 0.5 \). A \( K_{0.5} \) of 1.5 × 10^{-7} M and an \( n \) value of 3.1 were calculated from the fit.

DISCUSSION

Direct demonstration that anti-Met antibodies protect against dissociation of F1 from SCA 4-4-20 was obtained by using hydrostatic pressure as a tool to induce ligand dissociation. Previous studies that showed stabilizing effects of anti-Met antibodies were based on measurements of F1 dissociation from the antibody in the presence of excess fluoresceinamine (Weidner and Voss, 1991; Weidner et al., 1992, 1993). In those studies, the possibility that anti-Met antibodies influenced binding affinity of the antibody for fluoresceinamine could not be discounted. Using hydrostatic pressure as an external physical variable, ligand dissociation was induced (Weber and Drickamer, 1983; Weber, 1987, 1992), and thus the only species involved in the dissociation equilibrium were ligand, 4-4-20 antibody, and anti-Met antibodies.

Polyclonal anti-Met antibodies promoted a small release of F1 from SCA 4-4-20 at atmospheric pressure (Figs. 2 and 4). Such F1 release might be induced by small amounts of anti-idiotype antibodies that could be present in the polyclonal anti-Met preparation. Presence of anti-idiotype antibodies in the polyclonal anti-Met population and their possible participation in the formation of a large immune complex along with anti-Met antibodies have been previously suggested (Weidner et al., 1992). Although the polyclonal reagent was affinity-purified over a liganded mAb 4-4-20-Sepharose column, some anti-
idiotypic contamination could arise from binding to a small amount of unliganded mAb 4-4-20 also coupled to the Sepha-
bose. In this regard, it was interesting to note that mono-
clonal anti-Met was dependent on anti-Met concentra-
tions (Fig. 4). We considered the possibility that the absence of additive ef-
effects of monoclonal anti-Met could result from insufficient sen-
sitivity of the detection method since relatively low levels of dissociation were obtained under pressure using the Fl ligand (14–17% at 2.4 kbar) (Figs. 1–4). In order to increase the sen-
sitivity in assessing anti-Met effects, the pressure-induced dis-
sociation of the HPF-SCA 4-4-20 complex was studied. It was previ-
ously described that binding of HPF to mAb 4-4-20 occurs with lower affinity than binding of Fl (Bedzyk et al., 1992). Thus, if pressure-induced ligand dissociation depends on anti-
body affinity for the ligand, pressure should be more effective in dis-
sociating HPF than Fl from mAb 4-4-20. In fact, the in-
crease in fluorescence intensity corresponding to dissociation of HPF from 0.1 μM SCA 4-4-20 at 2.4 kbar was 45%, whereas, under the same conditions, Fl fluorescence increased only 14–
17% (Fig. 6). This result showed that the extent of ligand re-

![Graph 1](image1)

**Fig. 8.** Effect of monoclonal anti-Met antibodies on pressure-
duced dissociation of HPF from SCA 4-4-20. Pressure-induced dis-
sociation of 0.02 μM HPF from 0.3 μM SCA 4-4-20 was measured in the absence (○) or in the presence of 0.3 μM 1A4 (●), 0.6 μM 1A4 (■), 0.3 μM of 1A4 plus 0.3 μM 3A5-1 (▲), or 0.3 μM 1A4 plus 0.3 μM P1E11 (▲).

![Graph 2](image2)

**Fig. 9.** Binding of 2,5-Dns-3A5-1 to mAb 4-4-20. Fluorescence anisotropy of 2,5-Dns-3A5-1 was measured upon addition of unliganded mAb 4-4-20 (△) or mAb 4-4-20 bound to Fl (○) or mAb 4-4-20 bound to HPF (▲). The concentrations of each ligand shown in the abscissa correspond to either total antibody concentration in the case of unli-
ganded mAb 4-4-20 or to the total concentrations of hapten in the case of liganded mAb 4-4-20. The total concentrations of Fl or HPF were considered equal to the concentrations of Fl-mAb or HPF-mAb complex, since a 5-fold molar excess of antibody 4-4-20 sites was present. Anis-
rotropy was measured 2 min after each antibody addition as described under "Materials and Methods." The inset shows the plot of 2,5-Dns-3A5-1 anisotropy against free Fl-mAb 4-4-20 complex. The concentrations of the Fl-mAb 4-4-20 complex not bound to 2,5-Dns-3A5-1 (free Fl-mAb 4-4-20 complex) were estimated as described under "Results" using $r_1 = 0.035$ and $r_2 = 0.110$. The higher anisotropy limit was obtained by a sigmoidal fit to the data shown in closed circles in the main panel.
lease induced by hydrostatic pressure was inversely correlated with antibody affinity.

Using the HPF ligand, dissociation from 0.3 μM SCA 4–4–20 reached 80% at 2.4 kbar (Fig. 6). At this SCA concentration, it was observed that both polyclonal and monoclonal anti-Met antibodies protect against HPF dissociation (Figs. 7 and 8). However, despite the expansion in the range of ligand dissociation achieved by use of HPF, monoclonal anti-Met antibodies did not present additive effects against pressure-induced ligand dissociation (Fig. 8). Thus, the absence of additive effects of monoclonal anti-Met does not seem to be due to lack of experimental sensitivity and probably indicates that monoclonal antibodies, SCA–1, P1E11, 1A4, and 2C3, recognize overlapping metatopes. The fact that all these monoclonal antibodies tested recognize the L-chain of liganded mAb 4–4–20 (Weidner et al., 1993) is in agreement with this view. The possibility that two different monoclonal antibodies simultaneously bind to the F1-SCA complex but binding of only one is enough to promote maximal stabilization is not corroborated by the fact that additive effects are observed using polyclonal reagent, i.e., a heterogeneous population of anti-Met antibodies.

Hydrostatic pressure is known to promote dissociation of several oligomeric proteins (for reviews, see Weber (1987); Silva and Weber (1993)). Although the two variable domains in SCA 4–4–20 are connected by a peptide linker, pressure could interfere with the interactions between the two domains, promoting partial dissociation. However, previous studies have shown that pressure up to 1.6 kbar does not affect the overall structure of liganded SCA 4–4–20 as indicated by absence of changes in intrinsic fluorescence of the antibody in the range of 1 bar to 1.6 kbar (Coelho-Sampaio and Voss, 1993). Nevertheless, the same study proposed that release of Fl from SCA 4–4–20 was easier relative to release from mAb 4–4–20 due to the broader structural fluctuations that characterize the former complex. Therefore, the ability of anti-Met antibodies to protect against pressure effects does not appear related to stabilization of domain-domain interactions but possibly correlates with stabilization of structural dynamics of SCA 4–4–20 itself and/or interactions between SCA and the F1 ligand.

Among monoclonal anti-Met antibodies, monoclonal SCA–1 presents one of the highest capacities in delaying ligand dissociation from SCA 4–4–20 (Weidner et al., 1993). Furthermore, SCA–1, in contrast to the other monoclonal anti-Met antibodies studied here, does not cross-react with SCA 04–01 (Weidner et al., 1993), an anti-single-stranded DNA monoclonal antibody that possesses a similar light chain as mAb 4-4-20 (Smith and Voss, 1991). For the above reasons, we chose anti-Met antibody SCA–1 to study binding to liganded mAb 4–4–20. Direct measurements of the binding of 2.5-Dns-3A5–1 to liganded SCA 4–4–20 were not possible due to the fact that the decrease in Dns mobility upon binding of a 26-kDa molecule such as SCA 4–4–20 would not be sufficient to promote significant changes in the anisotropy of 2,5-Dns-3A5–1. On the other hand, binding of a 150-kDa protein such as SCA 4–4–20 to 2,5-Dns-3A5–1 was cooperative. Such cooperativity may be related to the bivalency of both ligand (F1-mAb 4–4–20) and antibody (3A5–1). Thus, binding of the first mAb 4–4–20 binding site to one of the mAb 3A5–1 sites might facilitate interaction between their second binding sites. In fact, cooperative binding might be involved in recognition of multivalent antigens by antibodies in vivo, since cooperative binding increases the effective avidity of the antibody for the antigen (Karush, 1978). The possibility that binding of anti-Met antibody 3A5–1 to the F1-mAb 4–4–20 complex gives rise to large immune aggregates cannot be discarded at this point. Further studies using Fab derivatives will be carried out in the future to quantify binding affinities for individual binding sites.

Using dissociation rate measurements, an apparent $K_d$ of $3.6 \times 10^{-8}$ M has been calculated for the dissociation of monoclonal anti-Met antibody 3A5–1 from the primary immune-complex Fl-SCA 4–4–20.2 This value is 4 times lower than the $K_d$ of 1.5 $\times 10^{-7}$ M reported here using Fl complexed to whole IgG 4–4–20. It is important to note, however, that the $K_d$ constant calculated does not take into account the significant cooperativity involved in the binding process. Such cooperativity, as discussed above, increases the effective affinity between the two antibodies.

Monoclonal anti-Met antibody 3A5–1 presented higher affinity for the metatype complex Fl-mAb 4–4–20 than for HPF-mAb 4–4–20. This difference could reflect different affinities involved in formation of the primary immune complex, since mAb 4–4–20 presents higher affinity for Fl than for HPF. In order to exclude this possibility, we calculated the effective concentrations of HPF-mAb 4–4–20 complex throughout the binding plot, using the $K_d$ of $6.5 \times 10^{-7}$ M reported previously for the association of HPF to mAb 4–4–20 in the absence of 3A5–1 (Bedzyk et al., 1992). Such concentrations were at least 80% of the total concentration of HPF (abscissa in Fig. 9) and became more than 95% above 0.1 μM HPF. Therefore, the significant difference observed between binding of the anti-Met antibody to each metatype complex reflects a real capacity of 3A5–1 to distinguish between the two complexes. HPF differs from Fl by the absence of a carboxyl group attached to the phenol ring. This carboxyl group forms a hydrogen bond with L32Tyr stabilizing binding of Fl to mAb 4–4–20 (Herron et al., 1989). Absence of such a hydrogen bond could increase conformational fluctuations undergone by the primary antigen-antibody complex perturbing recognition by the anti-Met antibody.

This work has shown that both monoclonal and polyclonal anti-Met antibodies protect against pressure-induced dissociation of Fl and HPF from SCA 4–4–20. Although protection is evident, a gradual increase in pressure eventually promotes ligand dissociation even in the presence of anti-Met antibodies. Whether the primary effect of pressure on the ternary complex Fl-SCA-anti-Met is to promote anti-Met dissociation or whether its primary effect is to promote Fl dissociation while the anti-Met is still bound are hypotheses that remain to be investigated. Such studies could lead to further understanding of the mechanisms involved in stabilization of antigen-antibody complexes by anti-Met antibodies.

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