Mechanistic Studies of the Effects of Anti-factor H Antibodies on Complement-mediated Lysis*

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We have recently reported that complement factor H, a negative regulator of complement-mediated cytotoxicity, is produced and secreted by most bladder cancers. This observation was exploited in the development of the BTA stat™ and BTA TRAK™ diagnostic assays, both of which make use of two factor H-specific monoclonal antibodies in sandwich format. Here we show that both antibodies exert interesting effects on the biochemistry of complement activation in vitro systems. Antibody X13.2 competes with C3b for association with factor H and strongly inhibits factor H/factor I-mediated cleavage of C3b, thereby evidently inactivating a negative regulator of complement; yet, the antibody strongly inhibits complement-mediated lysis as well. Conversely, antibody X52.1, which does not compete with C3b and has no effect on solution-phase cleavage of C3b, is capable of enhancing complement-mediated lysis of various cell types, including cancer cells, by over 10-fold. Our observations indicate that it is possible to deconvolute the biochemical roles of factor H in complement by means of appropriate inhibitors, a finding with potentially valuable implications for both basic research and cancer therapy.

Factor H, a soluble negative regulator of the complement system, is produced and secreted by most transitional cell carcinomas of the bladder (1–3). Complement factor H (FH)† is a 150-kDa protein whose structure and biological roles have been well described (Refs. 4–6; for descriptions of the complement system, see Refs. 7–11). We recently reported that a number of human cancer cell lines produce and secrete either FH or closely related FH variants with FH biochemical activity (1). In situ hybridization experiments have shown that bladder tumors also produce FH message, while normal bladder epithelium produces little or no message (12). However, the significance of this phenomenon with regard to both biochemistry and cancer biology remains to be established. It is not known, for example, whether complement is able to act against bladder cancer (transitional cell carcinoma or TCC), or whether secretion of FH enables the tumor to ward off the attack of complement. Still, the observation of FH expression in a high percentage of patients with even low grade TCC may indicate that FH plays an important role in tumor survival.

FH-mediated regulation is thought to be important in controlling inappropriate activation of the alternative pathway of complement (APC; Refs. 13 and 14). Briefly, the classical pathway of complement activation is generally antibody-dependent, while the alternative pathway is activated by the presence of negatively charged macromolecules with repetitive structures, such as mannans or bacterial cell walls. The central activating protein in both complement pathways is C3. A normal constituent of blood, C3 can be spontaneously activated by hydrolysis of an internal thioester bond, or can be selectively activated by the complement cascade (7, 9, 11). The activated form is designated C3b (or C3(H2O) for the spontaneously activated form). Activated C3 species direct other complement proteins to an appropriate target by associating either with specific receptors or with certain structures that constitute recognition motifs, such as the carbohydrates elaborated from the surfaces of yeast and bacteria (15). Activated C3 bound to a target cell or macromolecular structure provides both an anchoring point and a biochemical component of the “C3 convertase,” a complex of activated C3 units and Bb fragments (16). The C3 convertase has proteolytic activity which is absent or minimally present in the separate proteins; as a complex, the convertase catalytically activates further C3 molecules, leading to a cascade effect and eventually to formation of the membrane attack complex by the downstream complement proteins. In principle, complement can lyse any cell that exhibits a C3b target, including the normal cells of the host, but the process is ordinarily held in check both by solution-phase control of the concentration of activated C3 species and by regulatory proteins that act to suppress the cascade even after immobilization of activated C3. FH plays an important role in each form of regulation; it acts with factor I in cleaving activated C3 species to inactive forms, and it is also capable of causing dissociation of the constituents of the C3 convertase and thereby hindering autocatalytic activation of the APC.

There are a number of reports of up-regulation of membrane-bound regulators of complement activation in cancer (17–22). One model of expression of regulators of complement activation, including FH, in cancer holds that their up-regulation could enhance the ability of cancer cells to escape lysis by the immune system (immune surveillance; Refs. 23 and 24). Our interest in the biological basis of FH production and in the potential of FH as a therapeutic target has led us to investigate the effects of anti-FH monoclonal antibodies (mAbs) on complement activation and to probe certain biochemical details of
their mechanisms of action. In this report, we describe the effects of an unusual anti-FH mAb on complement-mediated lysis of both erythrocytes and cancer cell lines and analyze its mode of action using purified complement components and other cell-free systems. A serendipitous product of intensive hybridoma procedures, the X52.1 mAb, in addition to serving as a critical component of a diagnostic test for transitional cell carcinoma of the bladder, has allowed us to enhance complement-mediated lysis by up to 15-fold. However, the mAb evidently does not affect the solution-phase control mechanism by which FH reverses spontaneous activation in the bloodstream; instead our evidence indicates that X52.1 specifically protects the C3 convertase from disruption by FH. Two other mAbs with contrasting modes of action (X13.2, X46.3) are also described. If it proves possible to separate the biochemical processes of complement activation with single reagents, it is reasonable to hope for the development of new therapeutic strategies to treat both cancer and various infectious pathologies that take advantage of inappropriate suppression of complement activity.

**EXPERIMENTAL PROCEDURES**

**Materials**—Human complement serum, factor B-depleted human serum, zymosan, sensitized sheep erythrocytes, other miscellaneous chemicals, and ATP assay reagent (50 mM Tricine, 10 mM MgSO4, 1 mM EDTA, 100 µM dithiothreitol, 1 mg/ml bovine serum albumin, 66 µg/ml luciferase, 590 µM luciferin as reconstituted) were purchased from Sigma. C2-depleted serum was purchased from Advanced Research Technologies (San Diego, CA). Rabbit erythrocytes (RE) for activity assays of the APC were isolated from rabbit blood which was purchased from Colorado Serum Co., Denver, CO. Immobilized trypsin used to activate C3 was purchased from Pierce. Polyacrylamide gels for gel electrophoresis were purchased from Novex (San Diego, CA).

**Monoclonal Antibodies**—mAbs X13.2, X46.3, and X52.1 were generated by immunization of mice with the S-300 fraction of the urine of TCC patients and subsequent hybridoma procedures, which have been fully described (1). Control mAb MOPC-21 was grown from a hybridoma originally purchased from the American Type Culture Collection.

**Western Blot Characterization of mAbs**—The specificities of X52.1 and X13.2 were further characterized by Western blots of tryptic fragments of FH, transferred to polyvinylidene difluoride membranes. The mAbs were detected with an alkaline phosphatase-linked goat anti-mouse polyclonal antibody (human, serum, sheep, IgG, Intergen, Purchase, NY) and TMB substrate, and scanned with a Bio-Rad GE DC densitometer. The Profile Analyst II program was used to calculate molecular weights. Similar blots performed with other complement proteins as targets established that X46.3 is specific for the α chain of C3.

**Cell Lines**—HL-60, LS174T, Raji, HTB5, HTB9, T-24, and RT4 were obtained from the American Type Culture Collection and maintained attractively to culture media, followed by incubation at 37 °C for 45–90 min and 37 °C for 4 h. In initial experiments on the HL-60 and LS174T cell lines, cytotoxicity was measured by exclusion of trypan blue from live cells; live and dead cells were counted immediately after aliquots were taken for analysis and the ratio was determined. Subsequently the rapid glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerokinase/phosphofructokinase-linked fluorometric assay, with a limit of detection of approximately 0.03 human nucleated cell lysis, was developed (26) and used to measure lysis of Raji, LS174T, and three bladder-cancer cell lines (HTB5, T-24, RT4).

**Hemolytic Assays**—Hemoglobin-release assays were performed with RE (APC) or sensitized sheep erythrocytes (classical pathway). A typical APC assay contained 1/20 volume of complement serum, 6 mM EGTA, 4 mM MgCl2, and mAbs diluted in GVB to final volume before addition of erythrocytes, which were added in 0.5 volume. The EGTA and MgCl2 were mixed to a 3:2 ratio and titrated separately to pH 7.4 before mixing to a 20-fold solution in order to avoid proton-release effects in the complement reaction. Reactions were preincubated for 90–100 min prior to addition of erythrocytes. Pilot studies indicated that this preincubation enhanced the effect of X52.1 on the complement reaction, probably by allowing formation of a pool of activated C3; additional preincubation had little effect. The degree of enhancement was dependent on the age of the complement preparation. In our view this represents a reasonable model of the biochemical environment of a solid tumor, where any complement proteins present must have slowly diffused from a spatially removed blood vessel. RE or sensitized sheep erythrocytes, previously equilibrated in GVB at 2–3 × 108/ml, were added in 0.5 volume, and the reaction was incubated at 37 °C with mild shaking. Absorbance was measured by removing aliquots at timed intervals from reaction tubes on ice (to assure equal incubation time), centrifugation at 1000 × g for 3 min, and removal of cell-free supernatants to a microtiter plate, which was read at 405 or 410 nm.

**Lysis of Nucleated Cells**—Nucleated cells were lysed by addition of 10–50% complement serum (preincubated with or without mAb as above) to cell culture media, followed by incubation at 37 °C for 50 min to 4 h. In initial experiments on the HL-60 and LS174T cell lines cytotoxicity was measured by exclusion of trypan blue from live cells; live and dead cells were counted immediately after aliquots were taken for analysis and the ratio was determined. Subsequently the rapid glyceraldehyde-3-phosphate dehydrogenase/phosphoglycerokinase/phosphofructokinase-linked fluorometric assay, with a limit of detection of approximately 0.03 human nucleated cell lysis, was developed (26) and used to measure lysis of Raji, LS174T, and three bladder-cancer cell lines (HTB5, T-24, RT4).

**ELA**—mAbs were conjugated with alkaline phosphatase, yielding an average of two AP molecules per mAb molecule (1), and the conjugates were detected by measuring hydrolysis of p-nitrophenyl phosphate at 405 nm after performance of ELA by techniques previously described (27). EIA was used to measure lysis of Raji, LS174T, and three bladder-cancer cell lines (HTB-5, T-24, RT4).

**Measurement of Kd(app)**—Apparent dissociation constants of mAb-FH complexes were measured by direct titration in sandwich EIA (sEIA) format and the data were reduced by Equation 1 or 2, as described previously (27). Because of the possibility that directly immobilized FH antigens were not spectrally pure, we had not studied anti-human FH monoclonal antibodies (FHmAbs) in this context. A typical ELA experiment, measurement of Kd(app) for X52.1 was determined by immobilizing X13.2 for capture of FH, and vice versa. To measure the Kd(app) of X52.1, the X52.1-alkaline phosphatase conjugate was added in 2-fold serial dilutions over the concentration range 0–2.5 nM to a microrite plate coated with 1 µg/ml X13.2, which had been preincubated for 4 h with 2 µg/ml FH. The Kd(app) of X13.2 was measured by adding the X13.2-alkaline phosphatase conjugate (0–2.5 nM) to immobilized X52.1 (0.11 µg/ml) and FH as above. The lowest concentrations of coating antibodies that yielded a reliable signal were used to minimize ligand depletion. Nevertheless, such measurements cannot be assumed to yield accurate dissociation constants for the solution-phase complexes, for several reasons: 1) the alkaline phosphatase conjugate may not bind as well as the free molecule; 2) the dissociation constants of the two antibodies are convoluted in the result, since dissociation of either the X13.2-FH or X52.1-FH complex leads to loss of signal in each case; 3) the antigen immobilized in a microrite well and “displayed” by a coating antibody may be sterically hindered from forming the strongest possible association with the detection antibody. However, the actual Kd is likely to be stronger than the Kd(app) measured by these methods, since all of these effects tend to weaken the association. It is therefore fair to assume that the measured Kd(app) is an upper bound on the Kd of the weaker of the two associations. [FH] in the equation is the effective concentration of immobilized FH (which can be fit as a disposable parameter under ligand-depletion conditions), Amax is the observed absorbance value (or rate of change of absorbance—see below) with no added conjugate, and Amax is the fit asymptote of the absorbance data.
Two equations were used for reduction of the association data. Equation 1 is the Michaelis-Menten equation with an adjustment for the zero-ligand value. Equation 2 is similar, but contains a quadratic correction to account for the difference between the known, total quantity of added ligand (mAb conjugate) and the actual concentration of free ligand. There is a significant difference between the two only if the quantity of active, captured target (FH) is not negligible with regard to the size of the free-ligand pool and the $K_d$ (see Ref. 27 for more detail). In the data sets used for measurement of $K_{d,app}$, non-linear regression to Equation 2 yielded a fit target concentration that was indistinguishable from zero in all cases, indicating that ligand depletion was negligible.

$$A_{obs} = \frac{[Ab]/([Ab] + K_d) + A_0}{A_{max}} \quad \text{(Eq. 1)}$$

$$A_{obs} = \frac{[Ab] + [FH] + K_d - ([Ab] + [FH] + K_d)^2 - 4([Ab][FH])^{1/2}}{2[FH]} + A_0 \quad \text{(Eq. 2)}$$

We therefore used Equation 1 to calculate the reported $K_{d,app}$. The $K_{d,app}$ of X13.2 was determined either with single ELISA readings at 405 nm, or by taking readings at six time points and using the slope of linear regression with time to calculate $K_{d,app}$ for the other mAbs in this study.

$\delta$ 405 nm, or by taking readings at six time points and using the slope of linear regression with time to calculate $K_{d,app}$ for the other mAbs in this study.

$\alpha$ 20% complement.

$\beta$ 3% complement, 13 nM X52.1.

$\gamma$ 33% complement, 300 nM X52.1.

$\delta$ 33% complement, 500 nM X52.1. Degree of lysis enhancement was similar to that with puromycin-treated Raji, but absolute level of lysis was not $\geq 20%$. $\epsilon$ 3% complement, 22 nM X52.1.

$\zeta$ 20% complement.

RESULTS

Antibody Specificity—EIAs performed in the process of screening the antibody panels showed that mAb X46.3 was specific for the C3 α chain and exhibited no detectable cross-reactivity with FH. The specificity of X52.1 and X13.2 was previously determined (1); these mAbs do not cross-react with C3, C3-derived fragments or other complement proteins. They appear to be specific for human FH.

Mapping of Sites of Antibody Association—According to data from Western blots, mAb X52.1 associated with the 120-kDa and 48-kDa fragments of trypsinized FH, consistent with assignment of its primary site of association to SCR domains 15–20 of FH. mAb X13.2 associated with the same fragments, and also with the 38-kDa fragment reported to contain SCRs 1–5, as well as the factor I cofactor activity of FH (28). No secondary site of association for X52.1 was seen in Western blot experiments.

RT-PCR—Results of RT-PCR detection of FH (1) and DAF messages are presented in Table I.

Lysis of Nucleated Cells—In our initial complement tests with nucleated cells, we studied the effects of X52.1 on lysis of the promyelocytic leukemia line HL-60, using a dye-exclusion assay to measure cell death. The presence of X52.1 (22 nM) enhanced complement-mediated lysis of this cell line by 73%. Subsequently we developed an ultra-sensitive luminescent assay for cell death and membrane damage, which we used to observe an 11-fold enhancement of complement-mediated killing of puromycin-treated Raji cells by X52.1 versus the complement-only background (26). Untreated Raji cells were much less sensitive to complement-mediated lysis than puromycin-treated Raji, but X52.1 still enhanced the rate of lysis by approximately 12-fold (Table I). We also wished to extend the results to TCC cell lines. HTB-5, which expresses no detectable FH (1) and low levels of DAF (Table I), was very readily killed by complement, but no effect of X52.1 was seen, probably because the cells’ poor defense against complement leads to such a rapid rate of lysis that any enhancement is not quantifiable. RT-4, which expresses very high levels of FH, exhibited a variable response to complement, and again, it was not possible to identify a separate effect of X52.1 on lysis. However, T-24, which expresses intermediate levels of FH, was lysed by complement, and the effect was enhanced by X52.1. Table I presents a summary of the lysis data and effects of X52.1 by cell type.

Effects of Monoclonal Antibodies on FH/Factor I-mediated Cleavage of C3b in Purified System—Fig. 1 shows the effects of various monoclonal antibodies on the cleavage reaction in a...
purified system. In this system, factor I and FH form a complex that cleaves the C3b α-chain to the iC3b fragments of 68 kDa and 43 kDa (lane 2 versus lane 1). X13.2 (anti-FH), X46.3 and X87.2 (anti-C3 antibodies) strongly inhibit this process at 5.2 μM, as shown by the strong “C3b, α” bands and the low levels of the two iC3b bands in lanes 10, 4, and 8, respectively. 2.6 and 5.2 μM mAb X52.1 did not inhibit FH/factor I-mediated cleavage (lanes 5 and 6).

Hemolytic Assays—Results using both X13.2 and X52.1 with RE at the lysis target (29, 30) were in sharp contrast to those observed with purified complement components. X13.2 was seen to be a strong inhibitor of complement-mediated hemolysis. However, X52.1 enhanced hemolysis by as much as 15-fold (Fig. 2), although titration to higher concentrations revealed that the effects of this mAb were biphasic, with inhibition of cell lysis observed at higher concentrations. We subsequently performed a number of control experiments to rule out trivial explanations of the phenomena (listed in Table II).

**Table II**

| Complement source     | Activation pathway | mAb          | Effects of mAbs         |
|-----------------------|--------------------|--------------|-------------------------|
| Full human            | Classical          | X52.1, X13.2 | None                    |
| Full human            | Alternative        | 20 nm X52.1  | Up to 15-fold enhancement |
| Full human            | Alternative        | 1 μM X52.1   | Strong inhibition of lysis |
| Full human            | Alternative        | X13.2        | Strong inhibition of lysis at 3 nm |
| Full human            | Alternative        | Heat-inactivated X52.1 | None |
| Full human            | Alternative        | X46.3a       | Strong inhibition of lysis |
| Full human            | Alternative        | MOPC-21 (unrelated) | None |
| FH-depleted serum     | Alternative        | 20 nm X52.1  | Hemolysis seen; mAb had no effect |
| FH-depleted serum     | Alternative        | 1 μM X52.1   | Hemolysis seen; mAb had no effect |
| Factor-B depleted serum | Classical      | X13.2        | Rapid lysis; mAbs had no effect |
| Factor-B depleted serum | Alternative     | None         | No lysis                |
| C2-depleted serum     | Alternative        | 20 nm X52.1  | Up to 15-fold enhancement |
| C2-depleted serum     | Alternative        | 1 μM X52.1   | Strong inhibition of lysis |
| C2-depleted serum     | Alternative        | X13.2        | Strong inhibition of lysis at 3 nm |

a Anti-C3 antibody.
To investigate in detail the slight time-dependent shift in hemolytic enhancement seen in Fig. 2, we performed an experiment in which the profile of hemolytic rates versus X52.1 concentration was measured at five time points (Fig. 3). Rates are reported both directly and as corrected (normalized) to the quantity of intact RE remaining in the reaction (determined by complete lysis of the RE in H2O).

EIA—EIA in sandwich assay format very similar to that employed in the commercial diagnostic kits based on FH yielded the following results. C3b competes with X13.2 for association with FH (Fig. 4). Measured values of $K_{d_{app}}$ of FH-mAb complexes are as follows: X13.2, 325 ± 76 nM; X52.1, 181 ± 37 nM; X13.2 in the presence of 11.3 nM C3b, 1.21 ± 0.21 nM. Using a higher coating concentration of X13.2, it was also possible to observe a >5-fold increase in the apparent affinity of X52.1 for FH in the presence of C3b ($K_{d_{app}} = 202 ± 87$ nM versus $1.17 ± 0.42$ nM; Fig. 5), despite the fact that C3b competes for FH with X13.2. Because of the larger concentration of coating antibody, these data required a three-parameter fit to Equation 2 instead of the two-parameter fit permitted by use of Equation 1. Further studies conducted during initial characterization of the mAbs showed that immobilized C3b and the X52.1-AP conjugate form a very strong “sandwich” for FH ($K_{d_{app}} < 1$ nM), while the X13.2-AP conjugate in the same system yields a 20-fold weaker signal at a 4-fold greater concentration.$^2$

Secondary sites of association with FH were demonstrated for each mAb by sEIA performed in an homologous configuration, in which a single mAb is used along with its AP conjugate as both capture and detection reagent. These experiments were repeated with competition by varying concentrations of the other mAb (X52.1 for the X13.2 homologous format, and vice versa). $K_{d_{app}}$ was found to be in the range of 10 nM in each case (30–50-fold weaker than the primary sites). Competition by 93 nM or higher concentrations of X13.2 completely abolished association of X52.1 with its secondary site, indicating that this site is proximate to one of the X13.2 sites (see “Discussion”). Both mAbs exhibited strong affinity for FH captured by immobilized C3b.

Acceleration of Zymosan Fixation of C3b—A second documented biological role of FH, apart from its cofactor activity, is disruption of the C3 convertase (15). If X52.1 were capable of protecting this convertase from FH, then it should be possible to observe an increase in the rate of appearance of C3-related fragments on zymosan particles in the presence of X52.1, concomitant with more rapid disappearance of C3 and/or C3b from

$^2$ R. J. Kinders, unpublished observations.
solution. Figs. 6 and 7 show the increased rate of disappearance of C3b from solution with X52.1 present and the concomitant appearance of a 70-kDa fragment corresponding to the C3b β chain on zymosan particles (7).

DISCUSSION

The results of lysis studies with human cancer-derived cell lines were both promising and informative. Of the six cell lines studied, two (T-24, RT4) express both DAF and large amounts of FH, two (HL-60, LS174T) express DAF but not FH, and two (Raji, HTB5) express no DAF or FH. Ease of complement-mediated lysis was inversely correlated with expression of both DAF and FH (Table I), but it proved possible to enhance lysis with the anti-FH mAb X52.1 in one line from each group (T-24, HL-60, Raji). In sharp contrast, the X13.2 mAb, although an effective inhibitor of solution-phase FH activity, was ineffective against cellular targets.

mAbs X52.1 and X13.2 were tested further in various ways, including studies of cleavage of C3b using purified components, hemolytic assays with rabbit erythrocytes, ELISA, and analysis of zymosan-induced complement fixation. The rather surprising finding that X13.2, which strongly inhibits solution-phase cleavage of C3b (Figs. 1 and 8A), actually inhibited complement-mediated hemolysis was soon followed by the observation that X52.1, which has no effect on cleavage of C3b in the purified system, was a strong enhancer of hemolysis at concentrations as low as 3.3 nM (using 1:20 complement). These results strongly suggest that X52.1 acts by a mechanism other than preventing solution-phase cleavage of activated C3, and this hypothesis is further supported by the fact that the serum concentration of FH is >2 μM (31), or >100 nM at the 1:20 dilution employed in the hemolysis experiments. It is unlikely that a 13 nM antibody could inactivate a large enough fraction of a 100 nM soluble FH pool to lead to a 15-fold enhancement of lysis (Fig. 2). This unusual stoichiometry implied that there was a subset of the FH pool for which X52.1 had much higher affinity. Mapping of the sites of association of the mAbs by...
Westerns against tryptic fragments of FH demonstrated that both the X52.1 high affinity site and a low affinity site for X13.2 lie within domains 15–20 of FH; the high affinity site for X13.2 is probably within domains 1–5. Structural and biochemical evidence has shown that there are three sites of association for C3b on the FH molecule. Each of the sites may play a distinct role in complement control (32); it is therefore not surprising that antibodies which bind to different parts of the FH molecule appear to affect different modes of FH activity.

Since the activities of X52.1 are difficult to explain by invoking solution-phase mechanisms alone, and since the phenomena are not seen when FH is absent (Table II), the biochemically effective target of X52.1 in the lysis experiments may be the specific subclass of the FH population which is in association with activated C3 at the cell surface (i.e. as part of the C3 convertase). This could explain the unusual stoichiometry of the X52.1 lysis enhancement, especially if the mAb were found to have higher affinity for FH that is associated with C3b than for FH alone (modulation by long range interactions of the affinity of C3b for other ligands has been reported; Refs. 31–33). In fact a number of lines of evidence suggest that the target of X52.1 activity is FH associated with the C3 convertase. 1) X52.1 has no effect on solution-phase cleavage of C3b, yet accelerates complement-mediated lysis of diverse cell types by a mechanism which depends on the presence of FH. Apart from the FH-factor I-mediated cleavage reaction, FH is not known to have a role in complement other than its decay-accelerator activity in regulation of the C3 convertase (and the related C5 convertase). 2) X52.1 strongly accelerates activation of the APC, but has no effect on the classical pathway. This is consistent with identity of the C3 convertase as the X52.1 target, since it is specific to the APC, whereas the cofactor activity of FH may also involved in regulation of the classical pathway (13). 3) X52.1 accelerates both disappearance of C3b from solution (Fig. 6) and deposition of C3b fragments on zymosan particles (Fig. 7). The C3 convertase is the only known entity regulated by FH that would be expected to accelerate the fixation step specifically. 4) sEIA has demonstrated that the presence of C3b leads to an increase of at least 5-fold in the affinity of X52.1 for FH, despite the fact that C3b competes with FH for the coating mAb (Fig. 5). The actual magnitude of the affinity increase may be greater, because, first, EIA immobilization of a protein is an imperfect model for the cell surface, and second, the FH-C3b association is relatively weak by EIA standards (34); some of the C3b may therefore be lost during washing steps. This enhancement of X52.1-FH affinity by C3b explains how X52.1 could block the decay-accelerator activity of FH even at mAb concentrations too low to be effective against the pool of

![Fig. 7. Acceleration of fixation of C3b on zymosan by X52.1. Silver-stained zymosan pellets (see Fig. 6 legend). Lane 1, 10 min, no X52.1; lane 2, 10 min, 100 nM X52.1; lane 3, 15 min, no X52.1; lane 4, 15 min, 100 nM X52.1; lane 5, 20 min, no X52.1; lane 6, 20 min, 100 nM X52.1.](image1)

![Fig. 8. Proposed modes of action of mAbs in complement activation and control. A, solution-phase effects. X13.2 inhibits solution-phase cleavage of activated C3 (represented here by C3b); X52.1 has no effect. FI, complement factor I. B, schematic depiction of decay-accelerator activity of FH against the C3 convertase. C, in the presence of X52.1, FH still associates with C3b but evidently does not dissociate the convertase. The inhibitory modes of the mAbs are not shown.](image2)
free FH. 5) Finally, under the hypothesis that the biochemically effective target of X52.1 is FH in association with the C3 convertase, and given that X52.1 has an additional, uncharacterized inhibitory mode at a weaker site of association (descending limbs in Figs. 2 and 3, homologous format data above), one might expect to see a time-dependent shift in the kinetics of X52.1-assisted lysis as the population of the convertase increases (via well described mechanisms; Ref. 16), causing a shift in binding of X52.1 from the weaker, inhibitory site to the strong site on convertase-associated FH. Fig. 2 indicated to us that it should be possible to observe such a shift: between the 45- and 88-min time points, the ratio of aggregate lysis at 13 nM to that at 33 nM changes from 2.14 to 1.15, and it is clear from inspection that the progress curve is shifting to higher concentrations of X52.1.

We investigated the kinetics of X52.1 enhanced lysis in a further experiment in which five time points were taken. These data are presented in Fig. 3, before and after normalization to the number of intact RE remaining to illustrate the point that no important effects can be attributed to exhaustion of the “substrate” of lysis (RE), although the 312-min time point shows clear evidence that some other critical component is being exhausted at 20 nM, perhaps C3. The concentration of maximum enhancement of lysis by X52.1 has shifted to higher mAb concentrations at each time point. This is highly consistent with a model in which competition between the “enhancer” site and the weak inhibitory site is gradually resolved in favor of enhancement as the target of the enhancement mode, presumably the C3 convertase, increases in concentration. Fig. 8B portrays our proposed model of the complement-enhancing effects of X52.1.

The experimental results shown in Fig. 3 allow us to perform a rough calculation of the number of X52.1 target molecules present on a rabbit erythrocyte, yielding an estimate of \(-480,000\) fixed C3b molecules per RE during rapid activation.3 This number agrees reasonably well with the measurement made by Fishelson et al. of 380,000 deposited C3b molecules per RE under similar conditions (35).

It is encouraging that X52.1 is able to enhance complement-mediated killing of several types of cancer cells, and the observation that the mAb evidently acts against inhibition of the C3 convertase, but has no effect on the solution-phase reaction, implies that several steps of complement activation may be accessible to the biochemist who wishes to manipulate these pathways for either therapeutic or research purposes. It also bodes well for complement-dependent therapy in general, since it establishes the possibility that rapid activation may be enhanced without profound and undesirable effects on solution-phase control in the bloodstream. However, there is reason to believe that still better reagents may be found. Since the inhibitory activity of X52.1 at high concentration is likely to be due to association at a separate site on FH, it may be possible to develop an antibody or other reagent which shows even greater specificity for the “enhancer” site. Alternatively, phase display (36–38) or polysome selection (39, 40) in a site-subtractive mode might be used to improve the specificity of X52.1 itself. Nevertheless, our data have shown that the feasibility of using anti-FH reagents in cancer therapy may depend critically on the presence or absence of complement regulators other than FH in the targeted cells. It may in fact be desirable to develop panels of mAbs against several of the cell-surface complement regulators in hopes of identifying reagents capable of isolating other steps within the activation pathways. The most likely choices of an initial therapeutic mode using X52.1 or a similar reagent are either intravesicular therapy of bladder cancer or localized therapy of a fatal condition such as pancreatic cancer.

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