Analysis of the recognition mechanism of the alternative pathway of complement by monoclonal anti-factor H antibodies: evidence for multiple interactions between H and surface bound C3b

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Abstract The ability of the alternative pathway of complement to discriminate targets as either activators or non-activators is mediated by different binding properties of factor H to surface-associated C3b molecules. In the present study we have probed the interaction between H and C3b using five anti-H mAb. The binding sites of the mAb were mapped by Western blotting using both recombinant and trypsin-generated H fragments. Two mAb bound to CCP1 (90X, 196X), two to CCP5 (MRC OX24, 86X) and one to CCP8–15a (131X). At a molar ratio 2:1 of ^{125}I-H:mAb all tested mAb enhanced binding of H to both activator- and non-activator-bound C3b. At higher concentrations two mAb had an inhibitory effect on H binding to surface-associated C3b (OX24, 131X). Thus the mAb 131X inhibits H binding to surface-bound C3b but unlike OX24 it does not bind to the previously described C3b binding site within or near CCP4–5. These results indicate that there is an additional interaction site on factor H for surface-bound C3b.

Key words: Complement factor H; Complement pathway, Alternative; Complement activation; Dextran sulfate; Monoclonal antibody

1. Introduction

The alternative pathway (AP) of complement (C) acts as a first line defence mechanism against a wide range of targets [1]. Initiation of its activation is not based on any specific target-associated factor. Instead, the AP is continuously activated at a low rate in human plasma by spontaneous hydrolysis of C3 molecules [2,3]. AP activation is limited by regulatory proteins in the fluid phase and on non-activator surfaces but not on activating foreign targets ('activators') [4–6].

Factor H (H) acts as a crucial fluid phase regulator of the AP by inhibiting the formation and activity of C3-convertases [7,8]. H is also capable of discriminating between AP activator- and non-activator-bound C3b molecules on surfaces [9–12]. High affinity of H to target-associated C3b restricts formation of the AP C3-convertase by preventing the binding of factor B to C3b and by supporting dissociation of C3bBb and rapid cleavage of C3b by factor I [7,8,13,14]. High affinity of H to C3b is favored by cell surface components present on AP 'non-activators', e.g. by sialic acid residues on glycophorin A and glycosaminoglycans [10–12,14,15], and leads to down-regulation of complement activation. C3b on 'activators', i.e. on surfaces which lack the protecting surface elements, has a relatively low affinity for H. Thereby the surface-bound C3b molecules escape the AP regulatory control.

Because of the important role of H in discriminating between host and non-host structures, the binding sites and mechanisms of interaction between H and C3b on different surfaces are of great interest. Factor H is an elongated 150 kDa glycoprotein composed of 20 complement control protein domains (CCP) [16]. The main interaction site of H for C3b has been located within or near CCP4–5 in the N-terminal 34 kDa tryptic fragment [17,18]. Factor H also has a binding site for polyanions ('heparin binding site') in CCP12–15 [19], which has been proposed to be an important site for recognition of non-activators. The interaction of H with C3b appears to involve at least two regions in C3b spanning residues 1187–1249 [20] and 727–768 [21] but whether two or more reciprocal regions on H exist has not yet been examined.

To analyze the binding of H to activator- and non-activator-bound C3b we have tested the effects of a set of anti-H mAb on the interaction. Our specific goals in this study were to map the binding sites of four previously uncharacterized anti-H mAb, and to examine how the binding of five mAb to different sites on H affects the interaction between H and C3b. Our results provide the first evidence that binding of H to C3b is not restricted to one site on H. Multiple interactions between C3b and H may play an important role during target recognition by the AP.

2. Materials and methods

2.1. Reagents and cells

Trypsin (type I from bovine pancreas), heparin (average *m* 11 000 Da), soybean trypsin-chymotrypsin inhibitor (SBTI) and zymosan (Z) were obtained from Sigma Chemical Co. (St. Louis, MO). Na^{125}I was obtained from Amersham International plc (Amersham, UK). For working solutions the reagents and cells were dissolved into veronal-buffered saline (VBS: 3.2 mM barbituric acid, 1.8 mM sodium barbital and 146 mM NaCl; pH 7.35), GVB (0.1% gelatin in VBS) or BVB (0.1% BSA in VBS). Zymosan was used in assays after boiling (10 min) in VBS.

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Abbreviations: AP, alternative pathway; H, factor H; CCP, complement control protein unit; DS, dextran sulfate; Z, zymosan.

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2.2. Antibodies

BALB/C mice were immunized intraperitoneally with highly purified human factor H on two occasions. 4 days after the second immunization, a mouse was splenectomized and the splenocytes were fused with the X63 Ag8 HAT-sensitive cell line using a modification of the method of Köhler and Milstein [22]. Supernatants from the resultant clones were tested for antibody to factor H and antibody-positive hybridomas were subcloned. Thereafter supernatant from each clone was again tested by EIA on factor H, C3, C5, and factor B. Only factor H-positive clones were identified. Four of these mouse hybridoma cell lines producing IgG anti-H antibodies (86X, 90X, 131X and 196X) were grown in RPMI 1640 (Gibco Laboratories, Paisley, UK) supplemented with L-glutamine (2 mM; NordCell, Bromma, Sweden), penicillin (10 IU/ml), streptomycin (100 μg/ml) and 10% IgG-free heat-inactivated FCS (Gibco BRL, Gaithersburg, MD). Antibodies were purified from hybridoma supernatants by protein G affinity chromatography (Pharmacia, Uppsala, Sweden). Three mAb (86X, 90X, 196X) were found to be of the IgG_1 subclass and one mAb (131X) of the IgG_{2b} subclass. MRC OX24 (OX24) antibody (mouse IgG_1)[23] was a kind gift of Prof. R.B. Sim (University of Oxford, UK).

2.3. Complement components

C3 and factors B, D and H were purified from human plasma using methods previously described [11,12,24,25]. Purity of these proteins was examined by SDS-PAGE and was found to be >90%. Factor H and C3 were radiolabeled with $Na^{125}I$ using the Iodogen method (Pierce Chemical Corp., Rockford, IL). Protein-bound radioactivity was separated from free ^{125}I by using Sephadex G-25 columns (Pharmacia). The initial specific activities of ^{125}I-labeled H and C3 ranged from 0.16 to 1.25×10^6 cpm/μg and from 2.2 to 4.4×10^6 cpm/μg, respectively. Limited fragmentation of H with trypsin was performed as described earlier [19], using 750 μg/ml factor H, 1.5% (w/w) trypsin, 55 μg/ml heparin and 3% (per weight of H) soybean trypsin-chymotrypsin inhibitor. Cleavage patterns were analyzed by SDS-PAGE.

2.4. Recombinant H fragments

Cloning, expression and purification of the various recombinant proteins have been described elsewhere [29]. Briefly, specific primers were used to amplify fragments containing various CCP domains using the human factor H cDNA as a template. Amplified fragments were cloned into the baculovirus transfer vector pBSV-8His. Recombinant virus was generated and isolated by standard techniques and used to infect *Spodoptera frugiperda* cells (Sf9). For recombinant expression Sf9 cells were grown in FCS-free Express medium (BioWhittaker) and the recombinant proteins were purified by Ni^{2+}-NTA agarose chromatography [30,31], 120 h after infection. Purity of the recombinant fragments was examined by SDS-PAGE and found to be >95%.

2.5. SDS-PAGE and immunoblotting

SDS-PAGE was performed according to Laemmli [32], using 8 or 10–15% gradient gels and either reducing (tryptic H fragments) or non-reducing (recombinant H fragments) conditions. Gels were either stained with Coomassie Brilliant Blue R-250 (Serva Feinbiochemica GmbH, Heidelberg, Germany), silver-stained or used for immunoblotting. For immunoblotting the peptides were transferred from SDS-PAGE gel to a nitrocellulose sheet (0.45 μm; Schleicher and Schuell, Dassel, Germany) [33], which was subsequently blocked with 3 or 5% (w/v) BSA in PBS. Antibodies diluted in 3% BSA-PBS were used at 10–20 μg/ml (17 h at +22°C). After washes (3×10 min) alkaline phosphatase-conjugated rabbit anti-mouse IgG (Zymed Laboratories, San Francisco, CA or Jackson ImmunoResearch Laboratories Inc., West Grove, PA) was used as a secondary antibody (1:1000 dilution, 60 min at 37°C or 2 h at 22°C). After three washes the immunoblot was incubated for 5–15 min at 22°C with a substrate solution containing nitroblue tetrazolium (Sigma) and 5-bromo-4-chloro-3-indolyl phosphate (Boehringer Mannheim GmbH, Mannheim, Germany) each dissolved in dimethyl formamide (Merck, Darmstadt, Germany) and finally diluted into 0.1 M $NaHCO_3$ buffer (pH 9.7) containing 1 mM $MgCl_2$.

2.6. Factor H binding assays

C3b was coupled to SRBC or zymosan by using the AP C3-convertase amplification method [26,27]. The number of C3b bound to cells during coupling was measured by using ^{125}I-C3 in a simultaneous and analogous coupling procedure. The number of C3b molecules ranged between 1400–9700/cell on SRBC and 115000–176000/cell on Z. Binding of ^{125}I-H to Z-C3b or SRBC-C3b was analyzed as described previously [11,12]. To study the effects of mAb on H binding, the mAb were equilibrated (5 min, 22°C) with ^{125}I-H prior to addition of cells. To separate cell-bound and free radioactive proteins the mixtures were rapidly centrifuged through 20% sucrose as described previously [28]. Radioactivity in both pellets and supernatants was counted. GVB or BVB was used as buffer and all assays were performed in duplicate.

2.7. Radioimmunoassay procedures

Radioimmunoassay (RIA) was performed using Polysorp plates (Nunc, Denmark) with removable wells. Wells were coated with 80 μl of H or BSA at 10 μg/ml in VBS (17 h, 22°C). After removing unbound protein, wells were washed and incubated with 200 μl BVB-T (0.1% BSA, 0.1% Tween 20; 15 min, 22°C) to prevent non-specific binding. Wells were washed three times with VBS-T (0.1% Tween 20), followed by addition of anti-H mAb at various dilutions and ^{125}I-H (≈30000 cpm) in 1/3 BVB-T. After incubation (60 min, 30 or 37°C) the unbound proteins were removed and wells were washed four times with 1/3 BVB-T. Wells were counted separately and all assays were performed in duplicate.

3. Results

3.1. Mapping of mAb binding sites on factor H

The binding sites of five anti-H mAb on H were mapped by Western blotting using both recombinant and tryptic fragments of H. Recombinant fragments containing CCP units 1, 1–2, 1–3, 1–4, 1–5, 1–6, 1–7, 2–6, 13 and 19–20 were used (Fig. 1a). 90X and 196X mAb bound to all fragments containing CCP1, but not to the fragment containing CCP2–6. However, 196X bound most clearly to fragments CCP1–6 and 1–7. In addition, weak binding of 196X was seen to other fragments containing CCP1. OX24 and 86X bound to CCP1–7, 1–6, 1–5 and 2–6 but not to the other fragments tested. 131X did not bind to any of the recombinant fragments tested (Fig. 1). None of the tested mAb bound to fragments containing

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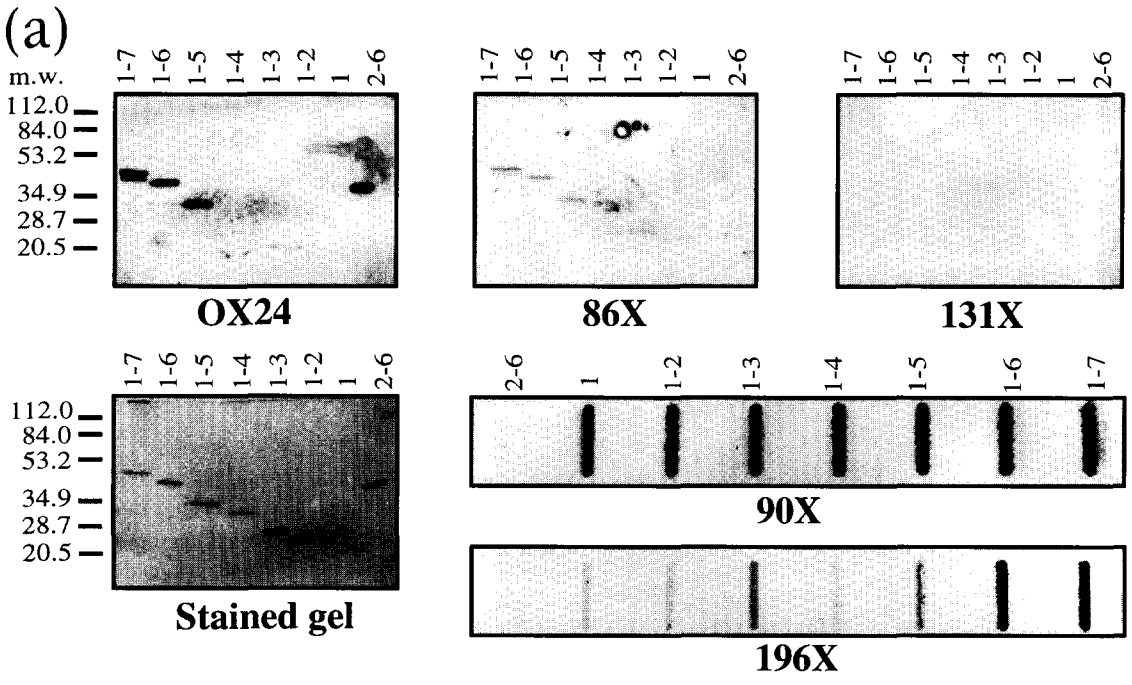
Fig. 1. Binding sites of five anti-factor H mAb. (a) Western blotting of recombinant H fragments with various anti-H mAb. Fragments (0.5 μg) were either separated by SDS-PAGE (10–15% gradient gels) under non-reducing conditions and electrotransferred to nitrocellulose sheets (86X, OX24 and 131X) or dot-blotted directly on nitrocellulose membranes (90X and 196X). Molecular weight standards are shown on the left, and numbers of CCPs in each recombinant fragment are shown on top of each lane or dot. (b) Western blotting of trypsinized H with various anti-H mAb. Factor H (750 μg/ml) was treated for 1 or 20 min with trypsin (1.5% w/w) in the presence or absence of heparin (55 μg/ml). Lanes: 1, native factor H; 2, 20 min treatment with trypsin in the absence of heparin; 3, 1 min treatment with trypsin in the presence of heparin; 4, 20 min treatment with trypsin in the presence of heparin. Samples were run in SDS-PAGE under reducing conditions and transferred to nitrocellulose sheets. Molecular weight standards are shown on the left and the molecular weights of distinct fragments of H are shown on the right. (c) A schematic diagram of trypsin fragments of factor H. Identity of the fragments obtained in the presence or absence of heparin, is based on chromatographic separation and N-terminal sequence analysis of the fragments [19]. Asterisk denotes previously uncharacterized trypsin fragment whose identity was deduced from its molecular weight and binding patterns of characterized mAb. (d) The deduced binding sites of the mAb used depicted on the elongated factor H molecule composed of 20 repeating CCP units. N-terminus is to the left. Deduced binding sites of the mAb were obtained from data in (a) and (b).


(a)
m.w.
112.0
84.0
53.2
34.9
28.7
20.5
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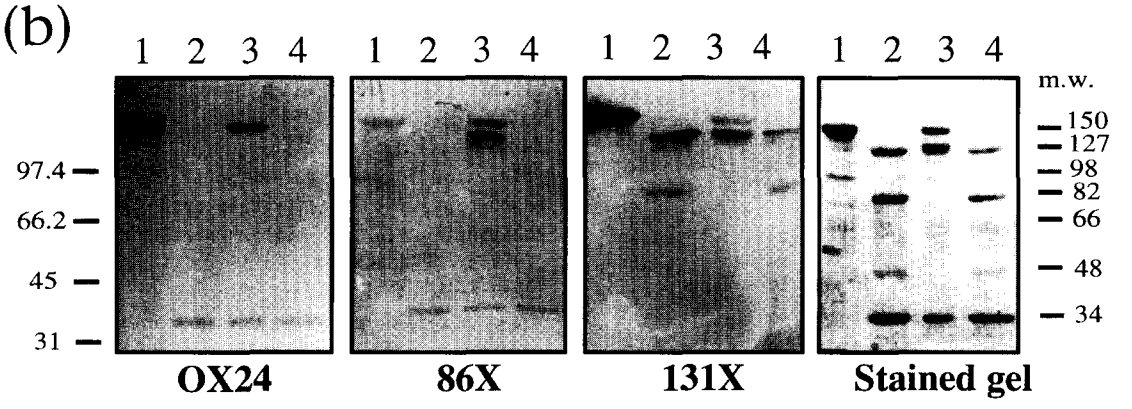
OX24
86X
131X
2-6 1 1-2 1-3 1-4 1-5 1-6 1-7
90X
196X
Stained gel


(b)
1 2 3 4
97.4
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86X
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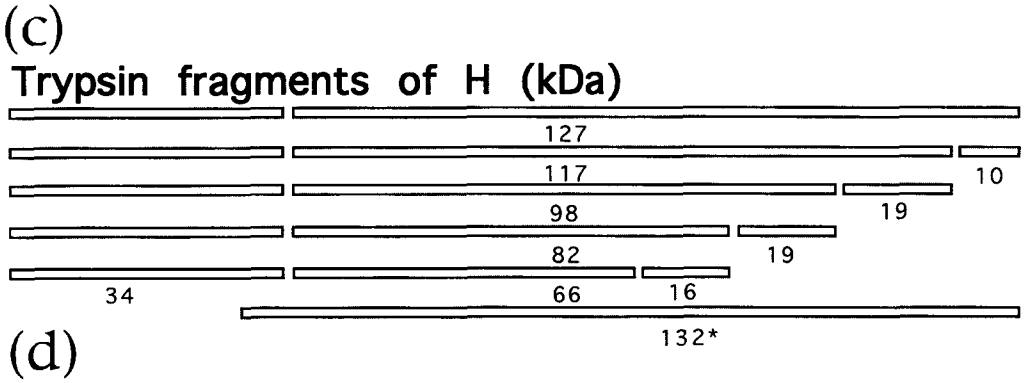
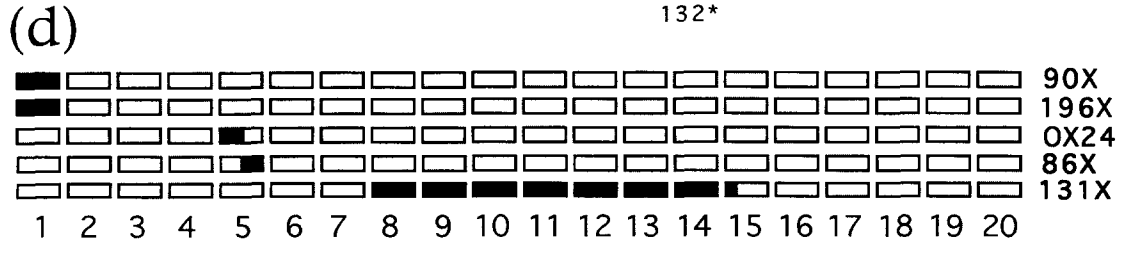
Stained gel


(c)
Trypsin fragments of H (kDa)
127
117
10
98
19
82
19
34
66
16
132*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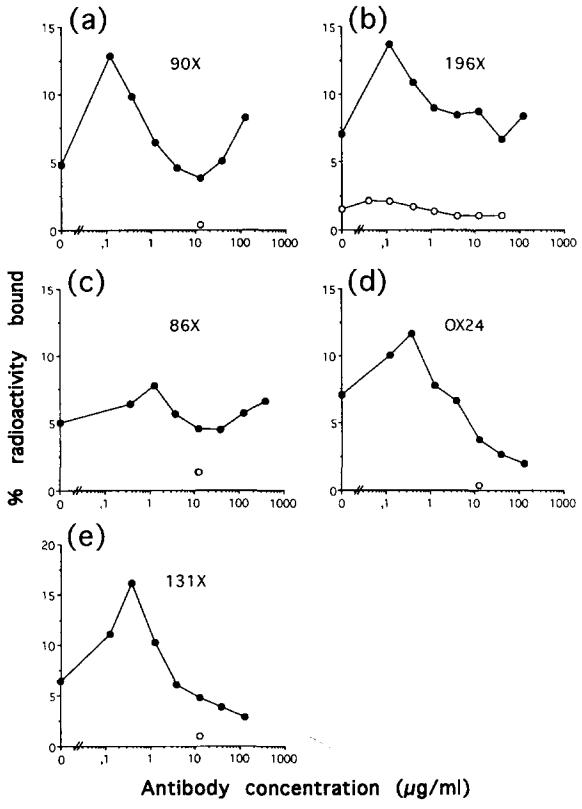

Fig. 2. Binding of ^{125}I-H to C3b-coated zymosan (●) or plain zymosan (○) in the presence of increasing amounts of anti-H mAb. Various mAb were incubated with ^{125}I-H (5 min at 22°C) in GVB or BVB before the zymosan particles ($6–12\times10^6$ particles/80 μl; 115000–175000 C3b molecules/particle) were added and the mixture incubated for 30 min at 37°C. Cell-bound radioactivity was determined after rapid centrifugation of cells through 20% sucrose.

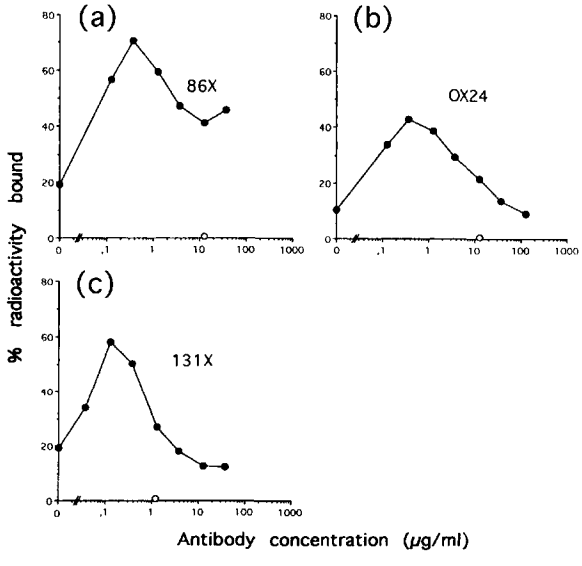

Fig. 3. The effect of increasing amounts of anti-H mAb on binding of ^{125}I-H to C3b-coated SRBC (●) or plain SRBC (○). The mAb and ^{125}I-H were preincubated together (5 min at 22°C), mixed with SRBC-C3b (3.3×10^7 cells/80 μl; 1400–9700 C3b molecules/cell) and incubated for 30 min at 37°C. Cell-bound radioactivity was determined after centrifugation through 20% sucrose.

CCP13 or CCP19–20 (data not shown). For mapping of the binding site of the 131X mAb well characterized tryptic H fragments were obtained as previously described [19]. The binding site was determined using a Western blot of H fragments separated under reducing conditions (Fig. 1b) and the previously published digestion pattern elucidated by N-terminal amino acid sequencing (Fig. 1c)[19]. The 131X mAb bound to the 127, 117 and 82 kDa tryptic fragments, and weakly to a previously undescribed 132 kDa fragment. 86X, 90X, 196X and OX24 bound to the N-terminal 34 kDa fragment as expected (only 86X and OX24 are shown in Fig. 1; the binding site of OX24 is the same as previously shown [34]). 86X bound also to the 132 kDa fragment, but not to the 127 kDa tryptic fragment. A summary of the mAb site analysis is shown in Fig. 1d.

3.2. *Effects of mAb on H binding to Z-C3b*

Five monoclonal anti-H antibodies (86X, 90X, 131X, 196X, OX24) were tested for their effects on H binding to surface associated C3b. At low concentrations (0.12–1.2 μg/ml) all the tested mAb showed an enhancing effect on H binding to Z-C3b (Fig. 2). The magnitude of the effect ranged from 1.6-fold (86X: 7.8 vs 5.0%) to 2.7-fold (90X: 12.9 vs 4.8%). The enhancing effect was no longer seen at higher concentrations (3–11 μg/ml), whereas at the highest concentrations (10–300 μg/ml) the effects of the mAb fell into three different categories: (1) two mAb (86X, 90X) showed an enhancing effect (Fig. 2a,b); (2) one mAb (196X) had no effect (Fig. 2c); and (3) two mAb (131X and OX24) inhibited binding of H to Z-C3b (Fig. 2d,e). The secondary enhancing effect varied from a 50% (86X) to a 116% (90X) increase as compared to the nadir. The maximum inhibitory effect varied from 55% (131X: 6.5 vs 2.9%) to 72% (OX24: 7.1 vs 2.0%) when compared to the basal level of H binding. Binding of H to plain Z in the presence of different mAb ranged from 0.4 to 2.0% of offered ^{125}I-H corresponding to 9–30% of ^{125}I-H bound to Z-C3b at the same mAb concentrations. The concentration of the mAb

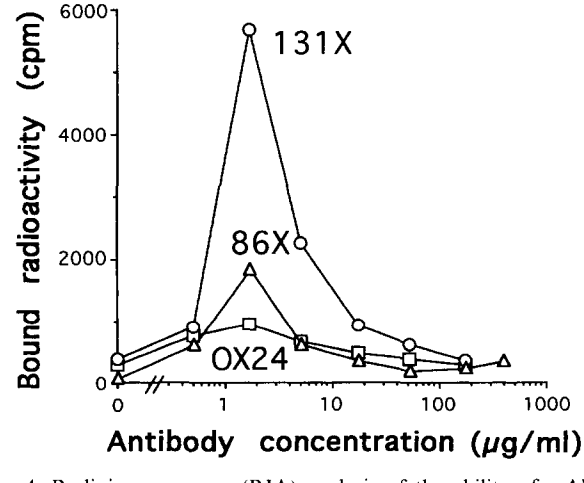

Fig. 4. Radioimmunoassay (RIA) analysis of the ability of mAb to cross-link solid-phase H and fluid-phase ^{125}I-H. After coating plastic plate wells with H or BSA, non-specific binding sites were blocked with 0.1% BSA. ^{125}I-H and increasing amounts of OX24 (□), 131X (○) or 86X mAb (△) were incubated in wells (60 min, 30 or 37°C) and the bound radioactivity was determined after four washes. Binding of ^{125}I-H to BSA coated wells was below 410 cpm/well.

did not significantly affect non-specific binding of H to Z (Fig. 2c).

3.3. Effects of mAb on H binding to SRBC-bound C3b

Binding of ^{125}I-H to SRBC and SRBC-C3b was examined in the presence of varying amounts of the anti-H mAb. On the basis of their different binding sites on H or different effects on H binding to Z-C3b three mAb (OX24, 86X and 131X) were chosen for the analysis. All three mAb showed a similar enhancing effect on H binding to SRBC-C3b as to Z-C3b at low mAb concentrations (Fig. 3), although the effect was smaller when using Z-C3b (Fig. 2). Also, at higher mAb concentrations the enhancing effect of 86X and the inhibitory effect of OX24 and 131X on H binding to SRBC-C3b were equivalent to that seen with Z-C3b. Binding of ^{125}I-H to plain SRBC was below 0.41% of total ^{125}I-H offered, compared with 10–20% binding to SRBC-C3b.

3.4. Cross-linking of H molecules by mAb

The ability of the different mAb (131X, OX24 and 86X) to form cross-linked dimers of H was examined by a sandwich-type radioimmunoassay using plates coated with H. Both 131X and 86X were found to link soluble ^{125}I-H to solid phase H in a dose-dependent manner (Fig. 4). A similar but somewhat weaker effect was seen with OX24. With each of the mAb an equilibrium peak effect was reached at a concentration of 1.7 μg/ml which corresponds to a molar ratio of 1:1.2 between the mAb and ^{125}I-H. At concentrations above 50 μg/ml only 86X had a weak secondary enhancing effect on H binding. This finding is compatible with results from experiments measuring H binding to cell-bound C3b.

4. Discussion

The special emphasis of the present study was to find out how different anti-H mAb affect the interaction between H and C3b – the key recognition event of the alternative complement pathway. The main observation was that mAb binding either to the N-terminal CCP5 domain (OX24) or middle region CCP8–15a (131X) of factor H were capable of inhibiting the binding of H to SRBC- and Z-associated C3b. This data suggests that the interaction between C3b and factor H is not restricted to one site only.

All tested mAb enhanced H binding to C3b-coated Z (Fig. 2) and SRBC (Fig. 3) at molar ratios of mAb to H ranging from 1:1.5 to 1:2.9. The sandwich RIAs verified that the mAb can crosslink two H molecules (Fig. 4). Thus, it is apparent that the low concentration enhancement effect, i.e. the increased avidity of H to surface-bound C3b, is explained by the ability of the antibodies to bind simultaneously to two distinct H molecules.

At mAb concentrations above 10 μg/ml two kinds of mAb effects on H binding, enhancing or inhibitory, were seen. 86X and 90X showed a second enhancing effect on H binding. The mechanism of this second peak of enhancement is not apparent, but could be explained e.g. by further cross-linking of H molecules by mAb binding to secondary low-affinity binding sites on H under conditions where the high-affinity binding sites are fully occupied. Because of structural similarity between the individual CCP domains it is possible that a single mAb can bind to more than one site on factor H. Interestingly, 196X mAb binds to CCP1 and not to CCP2–6 but the presence of CCP6 in addition to CCP1 clearly enhances the binding. It has previously been suggested that the N-terminal domains of H (CCP1–5) form a loop-like structure [34]. This model could explain the curious binding properties of 196X.

Among the mAb which bind to the N-terminal 34 kDa fragment of H (90X, 196X, 86X and OX24) OX24 was the only mAb inhibiting H binding to surface-bound C3b at higher mAb concentrations (>10 μg/ml). This mAb has previously been shown to bind to the region CCP4–5 [34] and to block H binding to RBC-bound C3b [23]. In the present study we found that OX24 binds to CCP5. We also found that OX24 inhibits H binding to Z-C3b at lower mAb concentrations than to SRBC-C3b (50% inhibition at ≈ 14 vs >125 μg/ml, respectively). Thus, it seems that either OX24 disturbs the binding site on H CCP4–5 for SRBC-C3b less than for Z-C3b or that on SRBC-bound C3b there is another binding site of H for C3b. Interestingly, another CCP5-binding mAb 86X did not inhibit H binding to surface-bound C3b. This may indicate that 86X mAb binds to an opposing face of the CCP5 domain.

The 131X mAb was found to bind to the middle part of H. It bound to a 82 kDa tryptic fragment (CCP6b–15a), but not to recombinant fragments of CCP1–7 or 13. The fact that 131X did not bind to a 66 kDa tryptic fragment derived from the 82 kDa fragment suggests that the epitope may be located within the 16 kDa fragment spanning the region CCP13b–15a. As binding to CCP13 was excluded by recombinant fragments, 131X probably binds to CCP14–15a. Interestingly, although 131X binds to the middle part of H it inhibits H binding to ZC3b and SRBC-C3b.

The mapping of mAb binding sites on H was performed by Western blotting and dot-blotting using both recombinant and tryptic fragments of H. It was found that all the mAb bound to H under both reducing and non-reducing conditions. The mapping with the recombinant fragments was performed under both reducing and non-reducing conditions after either transfer of fragments from an SDS-PAGE gel or direct dot-blotting on a nitrocellulose sheet. The data from all these blottings was consistent, i.e. the binding sites of the four mAb which bound to the recombinant fragments seemed mainly to be conformation independent and on the CCP1–7 there did not seem to be any additional conformation-dependent binding sites (only analysis under non-reducing conditions is shown in Fig. 1). In contrast, the mapping with tryptic fragments was performed under reducing conditions. The reduction was necessary to separate the trypsin-generated fragments from each other, but it also caused denaturation of the generated fragments and thus only the conformation-independent binding sites were explored. However, many antibodies can bind to conformational epitopes which are destroyed under reducing conditions and such epitopes on the CCP8–20 could not be detected by the mapping methods used.

Two main observations now suggest that there are at least two distinct sites on H important for its binding to surface-bound C3b: (1) despite mAb-induced blocking of a specific site on H during the enhancing effect (e.g. with OX24), the molecule still can interact with surface-associated C3b molecules, and (2) 131X mAb inhibits H binding although it binds to CCP8–15a far from the binding site for C3b within or near CCP4–5. In the two-binding-site model the N-terminal (within or near CCP4–5) binding site is affected by OX24 and the second binding site in the middle of H (CCP8–15a) is affected

by 131X. It is not yet known whether the second binding site interacts directly with C3b, non-activator-surface or jointly with both. However, we cannot exclude allosteric effects of the 131X mAb, i.e. binding of the mAb to one site could induce a thermodynamically stable conformational change at a functionally more important distant site, e.g. at CCP4–5.

Several results of earlier studies can be explained by the existence of two interaction sites between H and SRBC-C3b, e.g. the effects of polyanions [12,35,36] or various trypsin treatments of H [17,37] on H binding to surface-associated C3b. The binding sites on C3b for H have been analyzed by synthetic peptides and mAb, and at least two segments of C3b (residues 1187–1249 and 727–768) have been found to be involved in binding of C3b to H [20,21]. This fits well with the pattern of two distinct interaction sites between H and SRBC-C3b as suggested in the present paper. In contrast, some studies have suggested that H has only one binding site for C3b [34,38], but these studies did not address possible differences in H binding to fluid-phase, activator-bound and non-activator-bound C3b. It is possible that the putative second binding site on H for C3b is involved primarily during binding of H to non-activator-bound C3b. Therefore, these results may not necessarily be incompatible with those of the present study.

In conclusion, our study has shown that (1) there are at least two binding sites on H for a surface-C3b complex, and (2) all anti-H mAb can enhance H binding to surface-associated C3b by forming H dimers at equimolar concentrations. Multiple interactions between C3b and H could thus provide flexibility for the alternative complement pathway to discriminate between activators and non-activators.

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