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Binding Studies on Two Different Monoclonal Antibodies Raised Against CEA*

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Abstract—*Binding characteristics of two mouse monoclonal antibodies MA/1 and MA/200 raised to carcinoembryonic antigen (CEA) are presented. Double antibody radioimmunoassay and a solid phase assay confirmed that MA/1 binds weakly to CEA extracted from tumour tissue but strongly to CEA in patients' serum. Antibody MA/200 binds with high affinity to both 'tumour' and 'serum' CEA. Ligand–receptor relationships for MA/1 and MA/200 binding to both tumour CEA and CEA isolated from serum are presented from equilibrium binding data analysed by the method of Scatchard. Co-titration experiments also indicated that MA/1 and MA/200 and an established polyclonal anti-CFA bind to a similar sub-population of CEA or CEA-like molecules, but the binding data demonstrate that their respective binding sites are chemically different.*

INTRODUCTION

THE CELL hybridisation technique, introduced by Köhler and Milstein [1], is now widely used as a routine tool for the production of specific monoclonal antibodies. Application of this technique in the secreted tumour marker field is continuing to be useful both in the search for new markers [2, 3] and for resolving the antigenic specificities expressed by complex substances like CEA [4, 5]. It is now becoming widely accepted that the immunological heterogeneity of CEA in particular can give rise to antibody populations of different specificities [6] which could lead to inconsistencies between different CEA assays using different antisera [7] and could possibly limit specificity.

Use of monoclonal antibodies raised against CEA will undoubtedly provide a means to test the concept [8] that different immunological specificities may be expressed on different populations of CEA molecules and by different pathological conditions. In support of this concept, our earlier studies on radioimmunoassay using a monoclonal antiserum MA/1 [7] have indicated that these antibodies appear to bind to a different spectrum of CEA molecules or a subset of those recognised by at least two conventional sera.

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Earlier studies [9] have also indicated that MA/1 antibodies have a high specificity for a CEA-like antigen present in patients' serum but which reacts weakly with CEA purified from tumours. In the present study we have obtained further evidence in support of this finding and compared the binding characteristics of MA/1 with those of a new monoclonal antibody MA/200 raised against semi-purified CEA and a conventionally absorbed anti-CEA antiserum PK1G.

MATERIALS AND METHODS

Preparation of monoclonal antibodies

MA/1. The preparation of these antibodies has been described in a previous communication [9]. Briefly, BALB/c mice were immunised (s.c.) with 40 μg of highly purified CEA (R42) [10] in Freund's complete adjuvant followed 5 weeks later by a similar dose given i.v. The spleens from responding mice were excised and a red cell-free cell suspension prepared. The hybridisation method was the same as that previously described by Köhler and Milstein [1] using 10^8 spleen cells and 10^7 mouse myeloma cells. Cloning of antibody-producing hybrids was accomplished using both dilution and single-cell transfer. An ascites monoclonal antibody preparation MA/1 was obtained by intraperitoneal injection of 10^7 hybrid cells into mice.

MA/200. These monoclonal antibodies were

raised against semi-purified CEA as described above.

Screening of hybrids

Screening for both MA/1 and MA/200 antibodies was carried out using the double antibody method. The dilution of the second antibody for complete precipitation was pre-determined using heterologous mouse anti-CEA.

Binding of monoclonal antibodies to CEA

Antiserum titration curves. A checkerboard titration was used to determine the best dilution of rabbit anti-mouse Ig for the precipitation of monoclonal antibody-bound counts. The dilutions of rabbit anti-mouse which gave the optimum precipitation of bound counts at any given dilution of MA/1 and MA/200 were noted. Titration curves of MA/1 and MA/200 were set up by making two-fold dilutions over the range ×100–×51,200 in a constant dilution of normal mouse serum (at ×100). Fifty microlitres of each dilution of antibody were incubated with 200 μl of assay buffer and 50 μl of [^{125}I]-CEA. On the second day the bound counts were precipitated with 50 μl of rabbit anti-mouse (×20) and 50 μl of polyethylene glycol 6000 (PEG). The rabbit anti-mouse serum (at ×20) was at the pre-determined optimal dilution for precipitation for MA/1 and MA/200 in the normal mouse serum diluent when used at ×100. The concentration of the latter (×100) was adjusted to take into account the varying concentration of immunoglobulin in the MA/1 and MA/200 preparations.

The antiserum titration for MA/200 was carried out in the presence of (a) pooled normal human serum and (b) fresh serum from healthy individuals. In each case 100 μl of assay buffer was replaced by 100 μl of serum.

Competitive inhibition of binding

By CEA extracted from tumour tissue. Inhibition studies were carried out using MA/1 at ×1000 and MA/200 at ×12,000 diluted in ×100 and ×400 normal mouse serum respectively. Previous studies with MA/1 [9] have shown that inhibition of the binding of MA/1 to [^{125}I]-CEA was difficult, requiring large doses of antigen to achieve displacement of label. Nine two-fold dilutions of CEA in assay buffer were made, starting at 43,000 ng/ml for MA/1 and 2000 ng/ml for MA/200. Assay triplicates were set up in 3-ml plastic tubes with 100 μl of each CEA dilution, 50 μl of MA/1 or MA/200, 100 μl of assay buffer and 50 μl of [^{125}I]-CEA. At the same time zero antigen tests were carried out by replacing the CEA with buffer and non-specific binding determined by replacing the monoclonal antibody with buffer containing an equivalent amount of normal mouse serum. All assay tubes were incubated at 37°C for 16 hr. Fifty microlitres of rabbit anti-mouse Ig was then added to all tubes together with 50 μl of 10% PEG. The tubes were filtered on a Kemtek R.I.A. machine and counted after 4–6 hr. The dilution of the second antibody for MA/1 and MA/200 was ×10 and ×80 respectively.

By serum CEA. Inhibition curves for MA/1 and MA/200 were set up as just described except that CEA (G84) extracted from human serum was used as the unlabelled antigen. In this case the top standard for both antibodies was 1000 ng/ml as measured on our routine assay (PK1G). Similar inhibition curves were also set up for the two monoclonal antibodies using 'untreated' human serum containing CEA and immunopurified CEA (G84/1) from a pool of 22 patients with colonic cancer.

Self-displacement analysis and Scatchard binding data for MA/1 and MA/200

Concentrations of labelled CEA from ×1 to ×20 were used to obtain self-displacement data for both monoclonal antibodies. Fifty microlitres of each label concentration were incubated at 37°C with 50 μl of MA/1 or MA/200 at their respective dilutions of ×1000 and ×20,000 and 200 μl of assay buffer. After 16 hr the bound counts were precipitated with rabbit anti-mouse serum and PEG as decribed previously and the immunoprecipitates counted. Non-specific binding and total counts/min for each of the concentrations of label were also determined, the latter being measured on an L.K.B. 80,000 Gamma sample counter. From these data the percentage of label bound by the antibody at each label concentration was calculated. Extrapolation from a standard CEA inhibition curve, determined in the same experiment, was performed to obtain the amount of CEA in ng at each label concentration. A linear equivalence curve of CEA against label concentration was used to obtain the amount of CEA in 50 μl of label at its working concentration. Scatchard plots of the bound/free ratio against the total bound CEA B in ng/100 μl were derived for both MA/1 and MA/200 binding to both tumour CEA (R41) and CEA purified from serum. Affinity constants were calculated from the slope of the Scatchard line:

$$-K = \frac{\text{bound/free}}{B} \text{ (l/mol)}^{-1}$$

[11]. The volume used for this calculation was 300 μl—the assay volume of the primary antibody

reaction. The molecular weight for CEA was assumed to be 200,000.

Solid phase assay for monoclonal antibodies

CEA preparations R41 (120 μg) and M-12 (540 μg) were coupled to an aminocellulose solid support by a modification of the method of Moudgal and Porter [12]. For the assay 100 μl of a ×200 dilution of the immobilised protein was incubated at 37°C for 1½ hr with the monoclonal antibody used at a dilution of ×1000 for MA/1 and ×2000 for MA/200. One millilitre of assay buffer was added, the mixture centrifuged at 30,000 *g* for 10 min and the supernatant discarded. After washing with 1 ml of assay buffer the precipitates were mixed with 100 μl of radiolabelled rabbit anti-mouse Ig and incubated for a further 2 hr at 37°C. The tubes were then filtered and counted on the Kemtek 3000. Inhibition of binding by M-12 and R41 standard CEA in the above assay system was determined by the addition of the CEA during the primary incubation.

Competitive binding between MA/1, MA/200 and the polyclonal anti-CEA serum PK1G

Preliminary competitive binding experiments were set up in order to determine whether there were any structural similarities between the binding sites for MA/1, MA/200 and PK1G.

Blocking of MA/200 binding by PK1G. Two-fold dilutions of MA/200 in ×400 normal mouse serum were made to cover the range ×400–×51,200. An antiserum titration in assay buffer (titration A) was then carried out by incubating at 37°C for 16 hr 50 μl of each MA/200 dilution, 200 μl of assay buffer and 50 μl of [^{125}I]-CEA. At the same time four additional titrations were performed in which part of the buffer was replaced with 50 μl of normal goat serum [titrations (b) and (c)] or 50 μl of PK1G diluted ×440 [titrations (d) and (e)]. After the incubation the bound counts were precipitated with 50 μl of rabbit anti-mouse Ig at ×80 and 50 μl of PEG (all titrations), and additionally with 50 μl of horse anti-goat Ig at ×4 for titrations (c) and (e).

Blocking of PK1G binding by MA/200. In this case five titration curves for PK1G (a–e) were set up, ranging from ×400 to ×51,200. Titration (a) was made in assay buffer, titrations (b) and (c) were made with 50 μl of normal mouse serum added and titrations (d) and (e) were made with 50 μl of MA/200 at ×400 added. Bound counts were precipitated with 50 μl of rabbit anti-goat Ig at ×20 and 50 μl of PEG for all titrations, and additionally with 50 μl of rabbit anti-mouse Ig at ×80 for titrations (c) and (e).

Blocking of MA/1 binding by PK1G. For this experiment five titration curves were set up as described for MA/200 in section (a) above. Dilutions of MA/1 from ×100 to ×3200 were made in ×100 normal mouse serum and the bound counts precipitated with ×20 rabbit anti-mouse Ig. The competing antibody PK1G was used at a dilution of ×440.

Co-titration of MA/1 and MA/200. Titration curves for MA/1 and MA/200 were set up with doubling dilutions in the ranges ×80–×2560 and ×1600–×51,200 respectively. Doubling dilutions were also made from a mixture of MA/200 (×1600) and MA/1 (×80). All the above dilutions were carried out using ×50 normal mouse serum so that a dilution of ×10 for the rabbit anti-mouse Ig ensured optimal precipitation of bound counts. Fifty microlitres of each antiserum dilution was then incubated at 37°C for 16 hr with 200 μl of assay buffer and 50 μl of [^{125}I]-CEA. The bound counts were subsequently precipitated with ×10 rabbit anti-mouse Ig and 50 μl of PEG. After 4 hr at room temperature the precipitates were filtered and counted as described earlier.

Radiolabelling

[^{125}I]-*CEA.* Twenty microgrammes of CEA (R42) were labelled with 2 mCi of ^{125}I (IMS 30, Amersham International) by a modification of the chloramine T technique [13] to a specific activity of 20 μCi/μg. Free iodine was removed by gel filtration on Sephadex G-200 and each fraction (~40 drops) corresponding to the protein peak assessed for binding to MA/1, MA/200 and PK1G. The highest binding fractions were the same for each antibody. Two fractions were chosen on the descending part of the protein peak to avoid aggregates. A dilution of 1:200 (×½ label concentration) of the pooled fractions was used throughout this work except that a ×1 concentration was used for Scatchard binding experiments.

[^{125}I]-*Rabbit anti-mouse Ig.* One hundred and thirty microgrammes of rabbit anti-mouse Ig (Dako 2109) were labelled with 2 mCi of ^{125}I by the chloramine T method to a specific activity of 8–10 μCi/μg.

CEA preparations

Semi-purified CEA M-12 was prepared according to the method of Coligan *et al.* [14]. CEA-R42 was further purified by chromatography on Concanavalin A-Sepharose as described previously [10]. Con A fraction 2B was designated R42. CEA-R41 was similarly prepared to R42 from a different pool of 6 liver metastases of colonic tumour.

Serum CEA (G84). Serum from 11 patients with colonic cancer, each having more than 1000 ng/ml of CEA, was mixed with an equal volume of 2 M

perchloric acid. After stirring for 10 min the mixture was centrifuged for 1 hr at 76,000 *g* and the supernatant dialysed against saline for 3 days. The solution was concentrated to a volume equivalent to 1000 ng/ml of CEA as measured on our routine assay using the PK1G antibody. G84/1 CEA from a further 22 patients with colonic cancer was also isolated from the serum by perchloric acid extraction and affinity chromatography using an absorbed polyclonal anti-CEA antiserum. The CEA in the bound fraction was concentrated to a volume equivalent to 1000 ng/ml of CEA as measured by our routine assay.

Buffer. The assay buffer used throughout this work was 0.05 M sodium phosphate at pH 7.1 containing bovine serum albumin (Ig/l) and thiomersal (20 mmol/l). PEG = 10% polyethylene glycol 6000 in water.

PK1G. The preparation of this antiserum and the corresponding radioimmunoassay have been previously described [7].

RESULTS

The frequency of detecting viable hybrids using the double antibody screening test was very low. One well out of 360 containing actively growing cells in the case of MA/1 and 1 out of 300 in the case of MA/200 contained hybrids producing detectable CEA-binding antibody. These hybrids remained viable after cloning and sub-culture.

Titration curves of MA/1 and MA/200 (Fig. 1) illustrate the binding of the antibodies to radiolabelled CEA. At saturating concentrations of MA/1 approximately 25–30% of [^{125}I]-CEA was bound. By comparison, MA/200 bound 50% of label at high concentrations of antibody and was more typical of the binding of our conventional anti-CEA (PK1G) to the same label and also the monoclonal antibodies described by Accolla *et al.* [15]. The effect of normal pooled serum on the binding of MA/200 to [^{125}I]-CEA is also shown in Fig. 1. Surprisingly, diminution in binding of up to 40% was obtained with several specimens of pooled and individual sera from healthy volunteers. Inhibition studies using standard CEA preparations revealed important differences in the binding of MA/1 and MA/200 (Fig. 2). Thus in the case of MA/200 50% inhibition of bound label was achieved by 90 ng/ml of CEA whereas in the case of MA/1 the dose required to produce the same inhibition was 8600 ng/ml, confirming our previous report [9]. This contrasts with a dose of 40 ng/ml in our routine double antibody assay using the polyclonal serum PK1G. These differences have also been demonstrated in the solid phase assay using two different preparations of inhibiting CEA (Table 1). Thus amounts of purified CEA (R41) and semi-purified CEA (M-12) capable of producing 71.8 and 68.5% inhibition respectively of the binding of MA/200 failed to inhibit the binding of MA/1.

In contrast to the results with CEA derived from tumour tissue, an extract of serum CEA (G84) from patients with colonic cancer was an effective inhibitor in both systems. Thus approximately 100 and 30 ng of serum CEA were required to produce 50% inhibition of the binding of MA/1 and MA/200 to [^{125}I]-CEA respectively. Similarly,

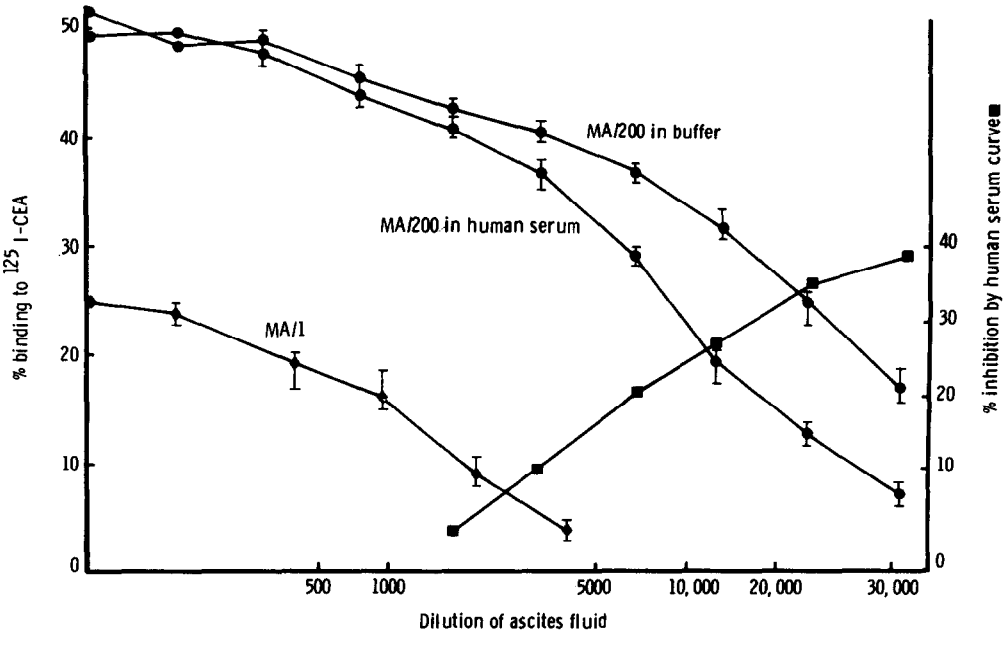

Fig. 1. Binding curves of MA/1 and MA/200 to [^{125}I]-CEA. Effect of normal human pooled serum on the binding of MA/200. Similar results were obtained when fresh serum from healthy individuals was used.

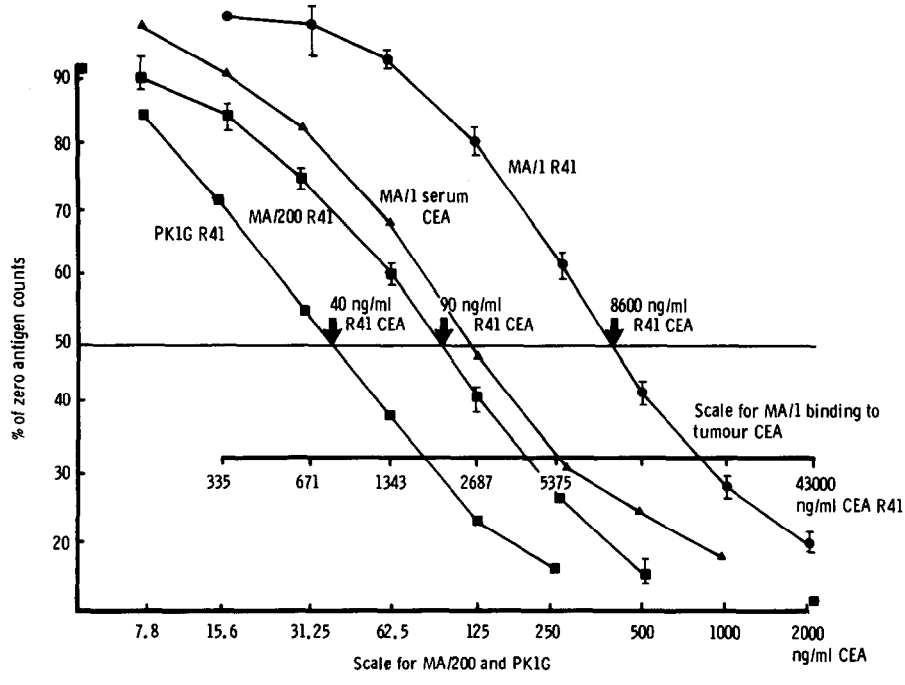

Fig. 2. Standard curves for the inhibition of binding of MA/1, MA/200 and PK1G to [^{125}I]-CEA. The figures refer to an amount of CEA as determined by our routine assay using PK1G antibody and a laboratory standard CEA. Inhibition of the binding of MA/200 by 'tumour' CEA (R41) and 'serum' CEA (G48) were superimposable. Note the extended scale for reading off the inhibition of MA/1 by 'tumour' CEA (R41).

untreated serum from a patient with colonic cancer and immunopurified CEA from a pool of 22 patients was also able to inhibit effectively in the MA/1 antibody system (Table 1a). The dose–response curves for CEA extracted from tumour tissue (R41) and serum from patients with colonic cancer were parallel for both monoclonal antibodies.

Table 1. Solid phase inhibition assay

	CEA	Dose (ng/ml)	Inhibition (%)
MA/200	R41	2000	71.8
	M-12	15600	89.0
	M-12	2000	68.5
MA/1	R41	2000	n.s.
	R41	86000	78.3
	M-12	15600	n.s.

n.s. = inhibition not significant.

Table 1a. Inhibition of the binding of MA/1, MA/200 and PK1G by different CEA preparations

CEA type		Dose of CEA to give 50% inhibition for each antibody MA/1	MA/200	PK1G
'Tumour' CEA	R42	7640	98	38
	R41	8600	90	40
'Serum' CEA	G84	100	30	44
	G84/1	92	86	37
Patients' serum		76	—	42

The figures depict ng of CEA as measured by our routine assay.

The percentage bindings of MA/1 and MA/200 to increasing concentrations of radiolabelled CEA are given in Table 2. In the case of MA/1 the binding was independent of the label concentration, as reported earlier [9], and indicates low binding affinity. The percentage binding of MA/200, on the other hand, diminished as the label concentration was increased. The amount of CEA in ng corresponding to the binding of MA/200 at each concentration of label was read off by extrapolation from a standard inhibition curve. The linear curve of concentration of label bound

Table 2. Percentage binding of antibodies MA/1 and MA/200 to increasing concentrations of [^{125}I]-CEA

Concentration of label	Total counts	Counts bound at ×1000	% bound
×1	205480	23736	11.5
×2	466550	48948	10.5
×3	587127	68728	11.7
×4	804214	93904	11.6
×8	1618230	190950	11.8
×0.5	116595	13525	11.6
×1	218160	24434	11.2
×2	413872	44284	10.7
×4	769493	75410	9.8
×10	1707480	138305	8.1
×20	3243849	152460	4.7

in ng against the label concentration (Fig 3) shows that 50 μl of label at ×1 concentration (see Materials and Methods) corresponds to approximately 0.8 ng of MA/200 binding CEA. The ligand-receptor relationships for MA/1 and MA/200 have been carried out using equilibrium binding data analysed by the method of Scatchard. In the case of MA/200 the Scatchard plot was a straight line (Fig. 4), as expected for a monoclonal antibody.

In the case of MA/1, since the binding was independent of label concentration we were not able to calculate the precise amount of CEA in the label bound by this antibody. However, for comparative purposes it is possible to get a good estimate of this from the percentage of label bound by MA/1 at saturation compared to that bound by MA/200 at the dilution of label used. Comparative Scatchard plots for the binding of MA/1 to both tumour CEA R41 and CEA

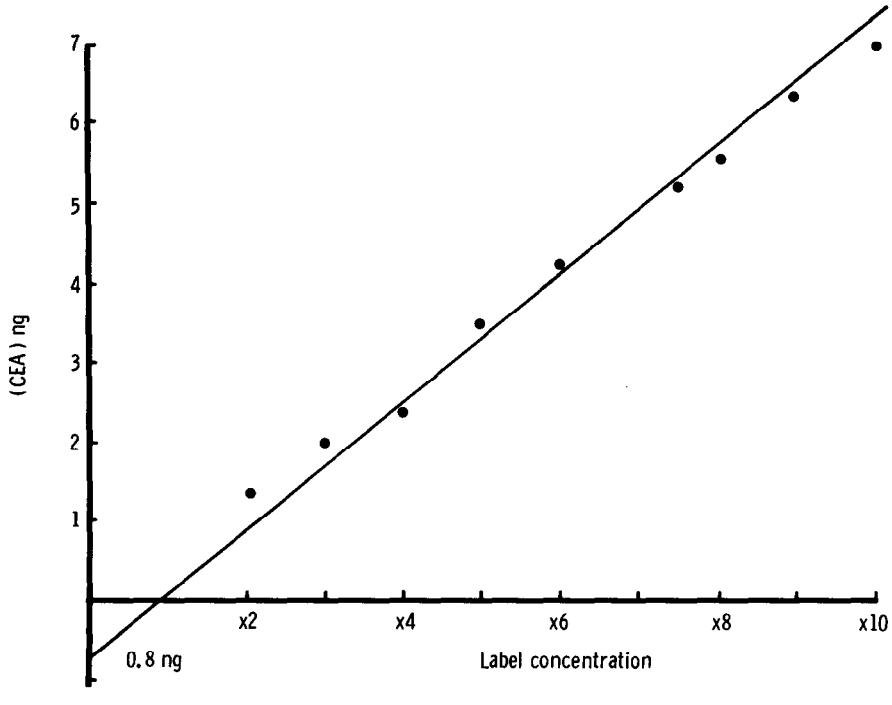

Fig. 3. Linear curve showing the extrapolated dose of CEA in [^{125}I]-CEA at different concentrations of label.

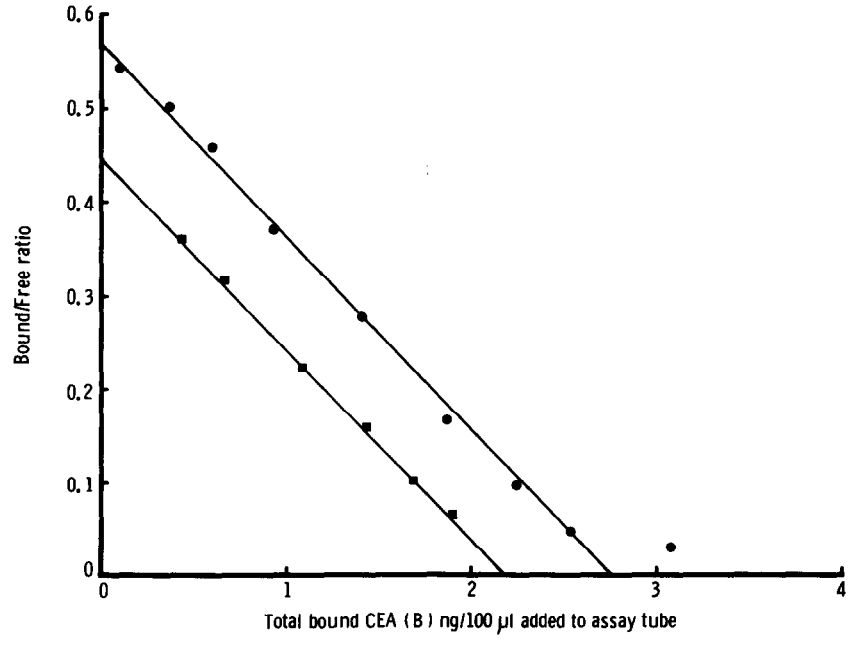

Fig. 4. Scatchard plot of the binding of MA/200 to 'tumour' CEA (R41) (•) and CEA in patients' serum (■). The total bound CEA at each dose level was calculated from the dose of cold CEA + 0.8 ng of CEA in the label.

immunopurified from serum are shown in Fig. 5 and have been calculated assuming 0.35 ng of label are bound by antibody in the absence of unlabelled CEA. In the case of the tumour CEA a straight line was obtained, indicating a homogenous population of antibodies and antibody binding sites. The slope of the Scatchard plot gives a measure of the affinity constant. It can therefore be seen that MA/1 has a lower binding affinity for tumour CEA than MA/200 (see Table 3). However, the Scatchard plot for the binding of MA/1 to CEA purified from serum yields two distinct slopes. In the case of a monoclonal antibody this must indicate the presence of at least two different classes of MA/1 binding sites in serum. One class reacts very weakly with MA/1 and in this respect is similar to the binding sites on tumour CEA, whereas the major sites bind with higher affinity to MA/1. The differences in affinity constant for MA/1 between the tumour and serum CEA (Table 3) help to explain the readiness with which CEA extracted from treated serum, or in untreated serum from patients with cancer, inhibits the binding of MA/1 antibodies to radiolabelled CEA. Moreover, the presence of only low affinity MA/1 binding sites in tumour CEA explains the necessity for high doses of tumour CEA standard to achieve inhibition in the MA/1 system. The affinity constants for MA/200 binding to CEA extracted from tumour (R41) and both purified CEA from treated and untreated serum are, however, similar (Table 3), showing that the respective MA/200 binding sites in these preparations are probably identical in structure and conformation.

Table 3. Binding data for MA/1 and MA/200

Antibody	CEA	Affinity constant K^* (l/mol)
MA/1	R 41 (tumour)	0.31×10^9
MA/1	G 84/1 (serum)	3.9×10^{10}
MA/200	R 41 (tumour)	12.6×10^9
MA/200	G 84 (serum)	12.2×10^9
MA/200	serum CEA	12.5×10^9
PK1G†	tumour CEA	5.6×10^9

*K has been calculated assuming a molecular weight for CEA of 200,000 and an assay volume of 300 µl. See Materials and Methods.

†This polyclonal antiserum has been shown to give a straight line Scatchard plot, indicating a largely homogenous population of antibodies.

The results of the blocking of MA/200 by our goat polyclonal anti-CEA PK1G are shown in Fig. 6. Binding of MA/200 over the range of dilutions (×400–×51,200) was not significantly different, whether the incubations were carried out in buffer or in the presence of normal goat serum, normal goat serum + horse anti-goat Ig or the goat anti-CEA PK1G. As expected, including both PK1G and its precipitating antibody horse anti-goat in the titration did produce high binding at each point on the curve, demonstrating the activity of PK1G in this experiment. These results show that the binding of MA/200 to CEA is not blocked by the presence of high concentrations of PK1G. In addition, the precipitated counts due to the binding of MA/200 at saturating levels are only marginally enhanced in the presence of PK1G and horse anti-goat Ig. This also suggests that most of the CEA protein

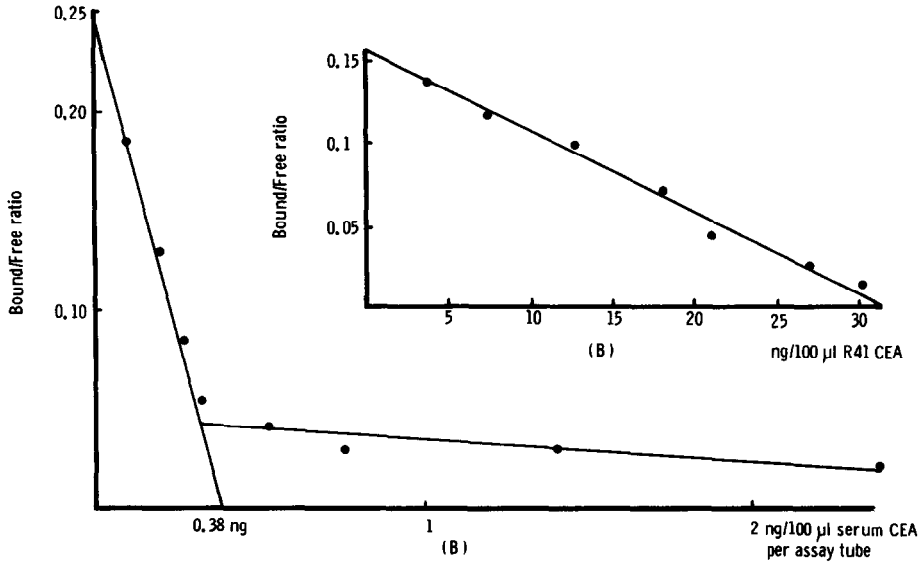

Fig. 5. Scatchard plot of the binding of MA/1 to CEA purified from serum (G84/1) and 'tumour' CEA (R41) (inset). The total bound CEA at each dose level was calculated using the estimated amount of CEA (0.35 ng) in the label (see text).

expressing PK1G binding sites also expresses MA/200 binding sites on the same molecule. If this was not the case a large enhancement of binding would be expected when the additional CEA-label–PK1G complex was precipitated. The results of blocking of PK1G by MA/200 is presented in Fig. 7. In this case, however, the presence of MA/200 anti-CEA antibodies partially blocked the binding of PK1G to CEA, whereas buffer or normal mouse serum did not. Moreover, a significant enhancement in overall binding was achieved when both antibodies were present, and this was shown experimentally when both precipitating second antibodies were used (Fig. 7). This suggests that more CEA glycoprotein is able to bind to MA/200 than is able to bind to PK1G.

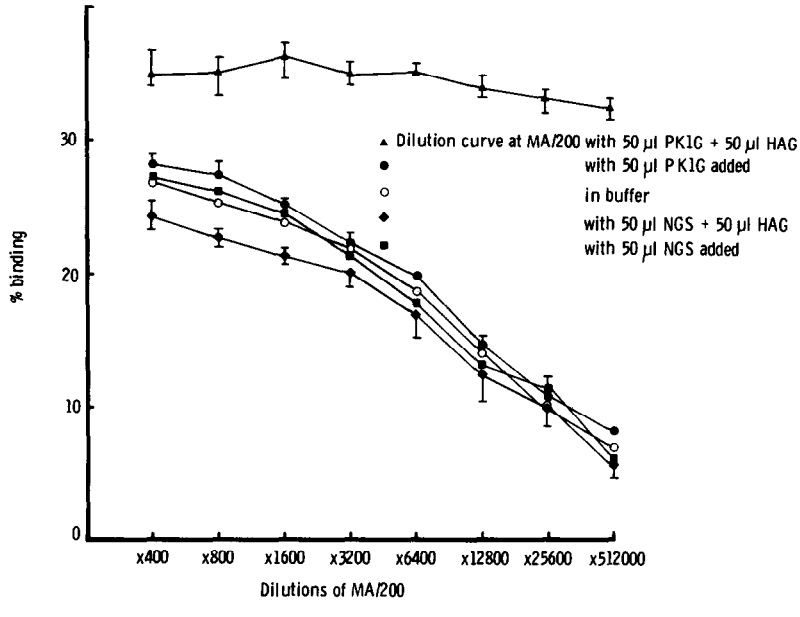

Fig. 6. Blocking experiment showing the effect of a high concentration of PK1G antibodies on the binding of MA/200 to [^{125}I]-CEA. HAG, horse anti-goat Ig serum; NGS, normal goat serum; RAM, rabbit anti-mouse Ig serum; NMS, normal mouse serum.

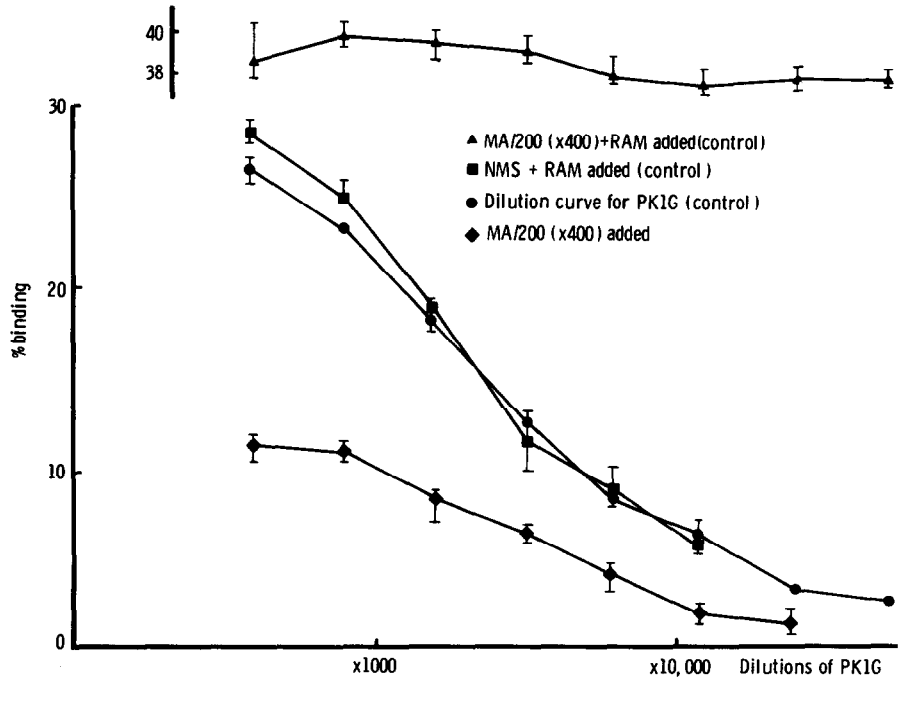

Fig. 7. Blocking experiment showing the effect of a high concentration of MA/200 on the binding of PK1G to [^{125}I]-CEA. The dilution curves for PK1G in the presence of normal mouse serum and buffer were identical—only the curve in buffer has been plotted. Abbreviations as for Fig. 6.

Furthermore, to account for partial blocking of PK1G binding by MA/200, there are probably protein structures on CEA which are common to the binding site for MA/200 and the binding sites of some of the component antibodies in PK1G.

Similar results were obtained with MA/1 (curves not shown). Thus the binding of these antibodies over the range of dilution from ×100 to ×3200 was not altered significantly by the presence of PK1G antibodies. However, on adding the horse anti-goat an enhancement of maximum binding from 24 to 38% was observed. This suggests that more CEA glycoprotein is able to bind PK1G than is able to bind MA/1. On the other hand, there was no augmentation of the binding of PK1G in the presence of MA/1 antibodies, showing that the CEA population expressing MA/1 binding sites also expresses the PK1G binding sites.

Competitive binding between MA/200 and MA/1 antibodies for labelled CEA has been studied by co-titration under conditions where optimal precipitation of bound counts were used throughout. In these experiments (see Table 4) the binding of MA/200 over the range of dilutions from ×1600 to ×51,200 was not significantly enhanced when co-titrated with MA/1 over the range of dilutions from ×80 to ×2560. This indicates that the respective binding sites for MA/200 and MA/1, which from other data presented cannot be identical, may have structural features in common but are at least expressed on the same molecular species, accounting for the observed result.

DISCUSSION

Despite the fact that the sera of all mice used for hybridisation had a high titre of anti-CEA, the frequency of detecting viable anti-CEA producing cells was only about 0.25% of the actively growing hybrids, indicating heterogeneity of the original hybrid population. Although the double antibody screening method used has a fairly wide tolerance to varying concentrations of primary antibody, there is an upper limit beyond which sub-optimal precipitation will ensue. Although unlikely, it is possible that some antibody producing cells could have been missed as a result. Double antibody screening methods or RIA using other precipitation methods [15] are sensitive to high affinity antibodies and this may also limit the number of antibody producing cells detected. Unpublished data from our laboratory concerning anti-HCG hybrids have indicated that more monoclonal antibodies may have been detected using the solid phase assay described in this paper. The use of highly purified radiolabelled CEA for the screening test and for the competitive binding studies described have enabled us to tentatively assign to MA/1 and MA/200 specificities to CEA rather than other proteins present in the immunising material. In addition, recent immunohistochemical studies (to be reported separately) have shown that both MA/1 and MA/200 produce identical patterns on immunoperoxidase staining of tumours to those obtained with our established anti-CEA serum PK1G.

The inhibition of the binding of MA/200 to CEA by human serum was a disappointing result which has made the assessment of this antibody for clinical assay more difficult. Current studies in our laboratory appear to suggest that components in human serum may interact with mouse protein in the binding assay. A solid phase 'sandwich' assay is being considered as a way round this problem.

The present study has substantiated our earlier evidence that MA/1 binds to a species of CEA more prevalent in serum than in tumour extracts. The binding of MA/1 to tumour CEA is of a lower affinity ($K = 0.31 \times 10^9$ l/mol) than its binding to CEA immunopurified from serum ($K = 3.9 \times 10^{10}$ l/mol), thus quantifying the specificity of MA/1 for serum CEA. The low affinity slope of the Scatchard plot (see Fig. 5) indicates that the 'tumour-type' CEA may also be present in serum but will not contribute effectively in radioimmunoassay. The binding site for MA/200,

Table 4. Co-titration of MA/1 and MA/200 binding to [^{125}I]-CEA under conditions of optimal precipitation of bound counts

MA/1		MA/200		MA/200 + MA/1*
Dilution	% binding	Dilution	% binding	% binding
×80	32.3	×1600	49.4	48.9
×160	16.7	×3200	44.8	46.1
×320	10.2	×6400	39.1	40.8
×640	5.1	×12800	28.2	33.2
×1280	2.7	×25600	18.3	22.2
×2560	1.9	×51200	10.5	13.6

*Corresponding dilutions of MA/1 and MA/200 were used for the co-titration. A constant mouse Ig concentration was used throughout.

however, appears to be immunologically identical in both tumour CEA and serum CEA ($K = 12.6 \times 10^9$ and 12.2×10^9 l/mol respectively). These results demonstrate that certain binding sites on CEA can be modified and account for differences between CEA in solid tumour tissue and serum. Other binding sites appear to be immunologically robust and more widely distributed.

Possible mechanisms for the expression of high affinity MA/1 binding sites on serum CEA could be glycosylation or modification of CEA on its passage from within tumour cells across the cell membrane, or modification or selective degradation in the liver and consequent enrichment of MA/1 binding CEA in the serum. The homogenisation and extraction process used in the isolation of tumour CEA could also destroy MA/1 binding sites which may be susceptible to proteases released as the tumour cells are disrupted, although this has not yet been tested. Work is currently in progress to isolate and characterise the MA/1 binding protein and establish its relationship to purified tumour extracted CEA.

Differences in the antigenic structure of CEA are potentially interesting and may lead to more sensitive or specific assays based on reagents derived from serum. Most current assays use 'tumour' CEA as a standard and to raise antibodies, yet these antisera might detect different immunological variants of CEA in or bind with different affinities to the tumour tissue derived standard compared to CEA in patients' serum. Evidence for this is apparent from different slopes of dose-response curves for tumour and serum CEA in some assays (G. T. Rogers, unpublished observation). In the case of MA/1 and MA/200 the dose-response curves for 'tumour' and 'serum' CEA are parallel, which is an essential requirement for radioimmunoassay which can be used for measuring CEA in both serum and other tissues and based on tumour CEA standards.

The solid phase assay described in this report was implemented to study the binding of MA/1 and MA/200 to CEA in a system independent of radiolabelled CEA. This eliminated the possibility that the labelling procedure itself might modify the binding of the antibodies and its competitive displacement. This proved not to be the case (cf. Fig. 2 and Table 1).

The competitive binding studies demonstrate that MA/1, MA/200 and the polyclonal serum PK1G recognise different binding sites on CEA. Moreover, there appears to be a main population of CEA which is able to bind all three antibodies. In addition, to account for partial blocking of the binding of PK1G by MA/200 there are probably chemical structures common to both of these sites. Despite the fact that the binding of PK1G is partially blocked by MA/200, the reverse appears not to be true, i.e. the binding of MA/200 is not blocked at all by PK1G. However, this apparent discrepancy may be resolved by the lower binding affinity of PK1G (5.6×10^9 l/mol) compared to MA/200 (12.6×10^9 l/mol). Similar blocking studies by Mitchell [4] have also shown that the binding of a monoclonal anti-CEA antibody is not blocked by a goat anti-CEA and therefore recognises a site on CEA not detected by the goat serum.

The co-titration experiment used to study the competitive binding between MA/1 and MA/200 was adapted from the methods reported by Fisher and Brown [16] and demonstrated that MA/1 and MA/200 must be binding to sites situated on the same molecule. The competitive inhibition and binding data, however, demonstrate that these two sites are apparently *immunologically* unrelated.

In conclusion, the results of this study have substantiated our earlier claim that MA/1 binds strongly to CEA in serum but weakly to CEA extracted from tumour. The kinetic data reported here help to quantify this difference in specificity. It is also evident that whereas CEA molecules share particular determinants, other satellite populations express only some of these determinants. CEA-like molecules therefore exhibit both intra- and inter-molecular immunological heterogeneity. Hybridomas raised against CEA are clearly able to produce low or high affinity antibodies which have the potential to isolate and characterise immunological variants of CEA and to assess their clinical value.

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