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A Monoclonal Antibody Against a CEA-related Antigen Expressed on HT29 Colon Tumour Cells*

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Abstract—*A new monoclonal antibody, that binds to CEA and with low cross-reactivity with NCA, has been raised to an antigen expressed on HT29 colon tumour cells. This antibody (H58) reacts strongly with high-molecular-weight protein (50×10^4) isolated from a crude plasma membrane preparation of HT29 cells as well as binding to purified CEA of molecular size (20×10^4) isolated both from those cells and liver metastases of colon tumour. H58 binds to an epitope sterically unrelated to the binding site of the previously described anti-CEA monoclonal antibody MA/1 and our routine anti-CEA polyclonal serum PKIG. Augmented binding of antibody to either the cell membrane preparation or conventional CEA can be achieved using a mixture comprising equal weights of specific immunoglobulin from H58 and MA/1. The value of solid-phase binding assays using microtitre plates for selecting potentially useful antibodies is discussed.*

INTRODUCTION

MONOCLONAL antibodies are becoming increasingly important both for detecting new tumour markers [1] and for the immunological characterisation of established markers such as CEA. Several new monoclonal antibodies have recently been prepared against CEA [2, 3] and their unique specificities are becoming apparent.

For our current studies we are exploring the use of tumour cell lines, plasma membrane preparations and preparations from serum to raise monoclonal antibodies against CEA. These methods should help preserve the expression of epitopes on CEA at the cell surface or on protein secreted from cells. The present paper reports on our initial attempt to produce monoclonal antibodies against CEA using as 'antigen' the colon tumour cells HT29 known to synthesise and secrete significant amounts of this glycoprotein [4]. This paper also documents a new antibody H58 which gives maximum binding to CEA or a cross-reacting antigen expressed by tumour cells. Purified preparations of H58 are being assessed for future application in radioimmunodetection of human tumours [5] and in tumour pathology using immunoperoxidase reactions.

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MATERIALS AND METHODS

Immunisation and fusion

Trypsinised HT29 tumour cells were suspended in culture medium (DMEM, Flow Laboratories) for 6 hr prior to immunisation. BALB/c mice received 2.6×10^6 viable cells in phosphate-buffered saline given i.p. followed by a similar dose given after 14 days and a final intravenous injection of 2.6×10^6 cells 20 days later. After a further 4 days spleen cells were fused with myeloma cells (P3–NS1/1Ag 4–1) according to the method of Köhler and Milstein [6]. Cloning by single-cell transfer was carried out after preliminary selection using the HT29 binding assay.

Binding

HT29 binding assay. Formalised HT29 cells were used at a concentration of 10^6 cells/ml. Cell suspension (100 μl) was mixed with 50 μl of culture fluid containing monoclonal antibody (sometimes diluted 1:4 in medium) and incubated at 37°C for 1.5 hr. Assay buffer (1 ml) was added and the mixture centrifuged at 30,000 *g* for 10 min. The supernatant was drained off and 100 μl of ^{125}I-labelled rabbit anti-mouse Ig (200,000 counts/

min) added to the residue. After 2 hr at 37°C the mixture was filtered on a Kemtek radioimmunoassay machine and the precipitate counted.

Binding assay using antigens adsorbed onto microtitre plates. Target antigens were selected as follows: HT29 membrane preparation (protein equivalent of 5×10^5 cells/well) (described later), protein secreted from HT29 grown in serum-free medium (protein secreted from approximately 10^6 cells over a period of 8 days/well), purified CEA (2 μg/well), normal colon (perchloric acid extract, 1000 μg/well), rectal tumour and colon tumour (perchloric acid extract of primary, 10 μg/well) pool of six liver secondaries of colon tumour (perchloric acid extract, 10 μg/well). Antigen preparations (50 μl) were dispensed into microtitre wells and left at r.t. for 16 hr and at 37°C for 4 hr. After washing gently ten times in tap water the plates were flooded with 1% gelatin in 0.01 M borate at pH 8 and left at 37°C for 4 hr. The plates were finally washed and left to dry. In the binding assay 50 μl of the hybrid culture fluid was added to the antigen wells in duplicate and left at 37°C for 4 hr. The plates were then washed in tap water and 50 μl of ^{125}I-labelled rabbit anti-mouse Ig (200,000 counts/min) added to each well. After 4 hr incubation at r.t. the wells were washed, cut up and counted. The rsults were expressed as the binding ratio:

$$\frac{C_{ab} - C_{med}}{C_{med}},$$

where C_{ab} = counts bound in presence of culture fluid and C_{med} = counts bound in medium. Monoclonal antibody MA/200, originally prepared in this laboratory [7], was used as a positive control.

For binding studies on purified H58, this method was also modified by omitting labelled rabbit anti-mouse and using labelled H58 instead. Negative controls for all binding assays used non-specific mouse immunoglobulin. Non-specific binding was also assessed using an ascitic fluid from a sham hybridisation in which spleen cells from a non-immunised BALB/c mouse were fused with myeloma cells as described.

Binding of H58 to [^{125}I]-CEA and inhibition. The titration curve and standard inhibition curves for H58 ascitic fluid were carried out as previously described [7].

Molecular weight studies

Binding of H58 to different molecular weight fractions of CEA. Semi-purified CEA was radiolabelled with ^{125}I to a specific activity of 8–10 μCi/μg as previously described [7] and fractionated on a column of G-200 using sodium phosphate buffer (0.1 M, pH 4.5, containing 1 M NaCl). The binding of H58 to each column fraction containing labelled protein was then determined using the double antibody method described [7]. In another experiment the crude membrane preparation was similarly labelled and fractionated (20 drops/fraction) on a calibrated Sephacryl S-300 column (0.9 × 60 cm). Fractions corresponding to labelled protein were diluted ten-fold and the binding of H58 and PKIG to each fraction determined.

By blocking. The dilution of H58 required to produce 95% of maximum binding by the microtitre plate assay was pre-determined. Using HT29 membrane preparation at an equivalent of 5×10^5 cells per well, this was shown to be 1:64. H58 binding at this dilution was then blocked with the HT29 membrane preparation or purified CEA as follows: HT29 membrane preparation (500 μg) or CEA (500 μg) was fractionated (8 drops/fraction) on a calibrated G-200 column. An aliquot (100 μl) of each fraction was then mixed with 100 μl of H58 (1:64) in gelatin-coated tubes and incubated at 37°C overnight. Of this 50 μl were then transferred to the microtitre target plate coated with HT29 membrane preparation and incubated at 37°C for 3 hr. After washing, labelled rabbit anti-mouse Ig was added and the plate incubated for a further 3 hr. The wells were then washed and counted. Blocking for each column fraction was calculated and the molecular weight profile plotted. The molecular weight of CEA components binding to PKIG was determined similarly.

Competitive binding between H58 and polyclonal anti-CEA

Blocking of H58 by PKIG. Ten two-fold dilutions of H58 in 1:50 normal mouse serum were made starting at a dilution of 1:50. Antiserum titration in assay buffer was then carried out by incubating at 37°C for 16 hr: 50 μl of each H58 dilution, 200 μl of assay buffer and 50 μl of [^{125}I]-CEA. For the competitive blocking, 50 μl of PKIG diluted 1:440 was added at each point of the titration instead of 50 μl of the buffer. An additional titration, including both PKIG and its precipitating antibody, horse anti-goat (1:4), was also set up. A titration in which the PKIG was replaced by normal goat serum (1:440) was used as a control. After incubation the H58-bound counts were precipitated with 50 μl of rabbit anti-mouse at 1:5 and 50 μl of PEG.

Blocking of PKIG by H58. In this case titration curves as described above were set up with ten dilutions of PKIG from 1:440 in 1:400 normal goat serum. For blocking 50 μl of H58 (1:100) was used with and without incorporating rabbit anti-mouse in the experiment.

Competitive binding between purified H58 and immunopurified monoclonal antibody MA/1

The binding of H58 and MA/1 used separately and also mixed in equal amounts was determined using both HT29 membrane (5×10^5 cells/well) and CEA (80 ng/well) as targets in the microtitre plate assay. In each case eight doubling dilutions were made in 1:50 normal mouse serum covering the range 200–1.5 μg/ml of IgG. Of each dilution of antibody, 50 μl was applied to the microtitre plates previously coated with the target antigens and incubated at 37°C for 4 hr. Bound antibody was detected using ^{125}I-labelled rabbit anti-mouse, as described previously. Normal mouse serum and purified IgG were used as controls. The binding for each antibody was corrected for non-specific binding produced by normal mouse serum.

Purification of H58

Using protein A–Sepharose. Five millilitres of H58 ascitic fluid was applied to a washed column of protein A–Sepharose (8 ml) (Pharmacia). Non-binding protein was eluted using 0.15 M phosphate-buffered saline, pH 8.0. The column was then eluted with 0.1 M sodium citrate buffer at pH 6.0 and subsequently with the citrate buffer at pH 3.0. The three fractions were dialysed against the phosphate buffer and concentrated to 20 ml. H58 activity in each fraction was determined by the binding to [^{125}I]-CEA.

By affinity chromatography using CEA–Sepharose. Ten millilitres of H58 ascitic fluid was filtered through a 0.45-μm microflow filter (Flow Laboratories) and applied to a column of CEA–Sepharose (22 mg CEA conjugated to cyanogen bromide–Sepharose, Pharmacia). The non-binding protein was eluted with three column volumes of phosphate-buffered saline and the column washed with a further three volumes of buffer. Bound H58 antibody was eluted with 2.5 M potassium iodide solution in the phosphate buffer and concentrated to approximately 1 mg/ml prior to dialysis against the phosphate buffer. The non-binding proteins from this column did not contain H58 antibody as determined by radioimmunoassay. For storage, purified H58 was kept at concentrations greater than 2 mg/ml to minimise aggregation.

Membrane extract from HT29 cells

Membrane preparations were obtained as described by Steplewski *et al.* [8]. HT29 cells (2.6×10^8) were washed in 3×15 ml saline. On microscopic examinations the cells were essentially whole with very little debris. The cells were resuspended in $ZnCl_2$ (10^{-3} M) containing a protease inhibitor (phenylmethylsulphonyl-fluoride) and incubated at room temperature for 30 min. The cells were then disrupted in a glass homogeniser and centrifuged at 200 *g* for 10 min. The supernatant was then recentrifuged at 30,000 *g* for 1 hr at 4°C and the sediment collected. The crude membranes were mixed in 15 ml of 3m KCl for 60 hr at 4°C and recentrifuged at 30,000 *g* for 1 hr. The supernatant was dialysed against phosphate buffer and concentrated to 24 ml using an Amicon PM 10 membrane.

Typing of H58

The immunoglobulin isotope was determined by immunodiffusion against rabbit antisera specific for IgG_1, IgG_{2a}, IgG_{2b}, IgG_3 and IgM (obtained from Miles Laboratories). Both cuture fluid and purified H58 were studied.

Labelling of purified H58

One hundred and sixty microlitres of immuno-purified H58 (224 μg) in phosphate buffer (0.15 M, pH 7.5) was mixed with 5 mCi ^{125}I at 4°C. One hundred microlitres of chloramine T solution (4 mg/ml) was added and the mixing continued for 1.5 min. Two hundred microlitres of L tyrosine (2 mg/ml) was then added and the mixing continued for a further 2 min. The labelled protein was separated on a pre-washed Sephadex G-200 column. H58 IgG_1 antibody was labelled to a specific activity of approximately 10 μCi/μg by this method.

Other materials

The CEA (R41) used in this study and the labelling procedure for CEA have been previously described [7]. MA/1 and PKIG are monoclonal and polyclonal anti-CEA antibodies and have also been described previously [7, 9]. Assay buffer refers to 0.05 M sodium phosphate at pH 7.1 containing bovine serum albumin (1 g/l). Purified NCA was kindly donated by P. Burtin. Rabbit anti-mouse Ig was labelled to a specific activity of 8–10 μCi/μg as described previously [7].

Immunohistochemical screening of culture fluids

Culture fluids were screened by the indirect immunoperoxidase technique using peroxidase-labelled rabbit anti-mouse Ig. Sections of colon carcinoma and the following normal tissues: colon, breast, skin, kidney, liver, lymph-node, stomach and lung, were used.

RESULTS

Hybridising and testing

From the 288 wells set up 198 actively growing hybrids (69%) were obtained for primary screening, and of these 61 (31%) were positive in the HT29 cell binding assay. Culture fluids from 13 hybrids bound to CEA but only six remained

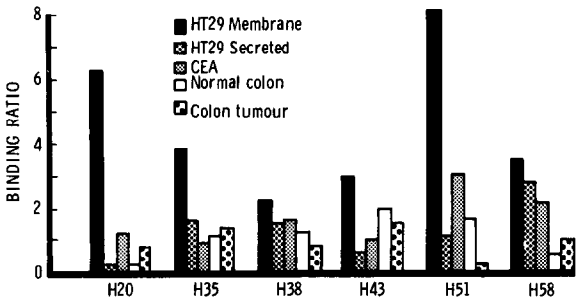

Fig. 1. Binding ratios of six CEA-reactive supernatants to the following targets using the microtitre plate assay: H29 membrane preparation (5 × 10⁵ cells/well), HT29 secreted protein, CEA (2 μg/well), normal colon extract (perchloric acid extract, 1000 μg/well) and rectal tumour extract (perchloric acid extract, 10 μg/well).

viable after twice cloning and were further tested by the microtitre plate assay (Fig. 1). These were designated H20, H35, H38, H43, H51 and H58.

H38, H51 and H58 showed the strongest binding to CEA. These antibodies also bound strongly to the H29 membrane preparation and secreted protein targets. H38 and H51 bound relatively strongly to normal colon by this assay and were rejected. H20 showed very strong binding to HT29 cells and the membrane preparation and weak binding to normal colon. Preliminary histological screening using a range of normal tissues and colon tumour, however, showed that with the exception of H58, all the antibodies (including H20) reacted equally with antigens expressed on normal colon cells as well as colon tumour cells. In contrast, H58 showed a staining pattern of the luminal surface with cytoplasmic staining of malignant glands with no background staining. Very weak staining of normal colonic mucosa was observed typical of specific anti-CEA antisera.

Purification of H58

The yield of H58 immunoglobulin after purification on the CEA–Sepharose column was approximately 300 μg of specific antibody per ml of ascitic fluid. Of the purified antibody, 92% was capable of specifically rebinding to the column. The isotype of the purified antibody was IgG_1. Partially purified H58 could also be obtained by affinity chromatography on protein A–Sepharose where it was eluted using pH 3.0 citrate buffer, insignificant activity by radioimmunoassay being present in the fractions eluted at higher pH.

Binding of purified H58 to solid phase target antigens

In addition to the binding described in Fig. 1, similar binding patterns were obtained for similar targets using immunopurified radiolabelled H58 or immunopurified H58 and radiolabelled rabbit anti-mouse Ig (Fig. 2). The dilution curves (Fig. 3) illustrate the specific binding of decreasing amounts of radiolabelled H58 to various solid-phase targets. Approximately 80% of the labelled H58 was bound to the HT29 membrane preparation at dilutions greater than 1:3200; however, at lower dilutions the percentage bound dropped significantly, indicating saturation of the antigen sites on the target.

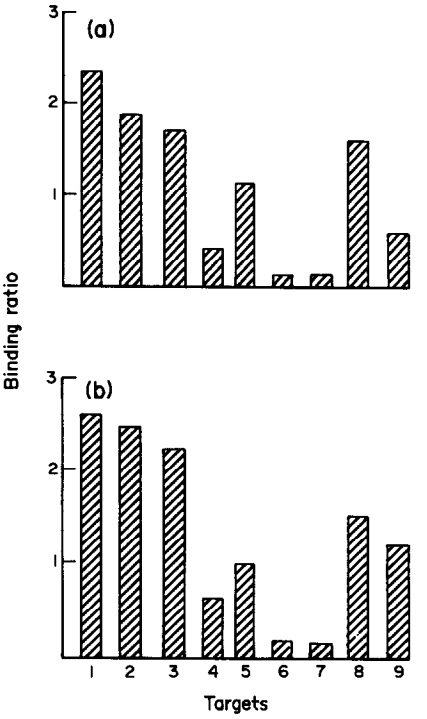

Fig. 2. These figures compare the binding of (A) H58 supernatant after prior blocking of the target with normal mouse serum and (B) immunopurified H58 to the following target antigens: (1) HT29 membrane; (2) HT29 secreted protein; (3) CEA; (4) normal colon extract (1000 μg/well); (5) colon tumour extract (10 μg/well); (6) normal human serum; (7) normal human liver extract; (8) colon tumour (pool of six tumours extracted with perchloric acid, 10 μg/well); (9) rectal tumour (10 μg/well).

Binding of H58 to [^{125}I]-CEA

Using the optimised double antibody assay over 60% of radiolabelled CEA was capable of binding to H58. Fifty per cent inhibition of bound labelled CEA was obtained with 500 ng of standard CEA and 370 ng of CEA isolated from serum, indicating a rather low binding affinity (4.6×10^7 l/mol by Scatchard analysis) for these particular CEA preparations. Cross-reactivity of H58 with purified NCA was less than 0.3% as determined by standard inhibition compared to CEA.

Molecular weight of H58 binding protein

Preliminary experiments using radiolabelled fractions of different molecular size prepared from semi-purified CEA suggested that H58 reacts

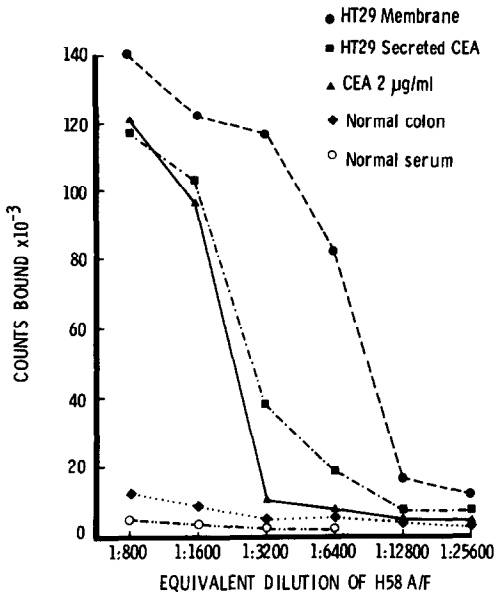

Fig. 3. Doubling dilutions of immunopurified H58 were made in 1:50 normal mouse serum. (These dilutions were made to have an equivalent antibody reactivity corresponding to dilutions of 1:800–1:25600 for H58 ascitic fluid.) 50 µl of each dilution were then applied to the coated microtitre plates and bound antibody determined with labelled rabbit anti-mouse Ig as described in Materials and Methods.

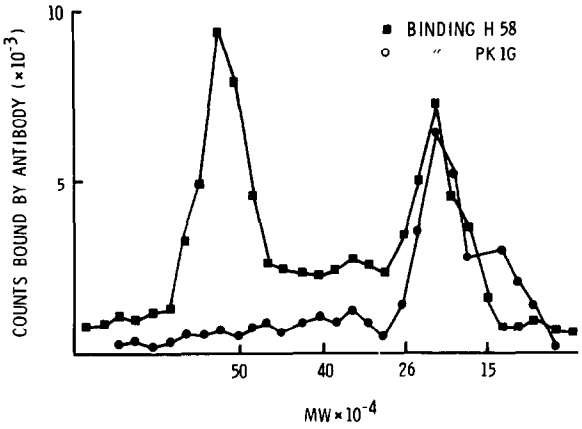

Fig. 4. Binding of H58 and PKIG to radiolabelled HT29 membrane preparations after fractionation of the latter on a calibrated Sephacryl S-300 column.

predominantly with a high-molecular-weight component of semi-purified CEA. Thus, in contrast to PKIG, which bound more strongly to CEA in the molecular weight range 180,000–200,000, H58 gave maximum binding with a species of labelled CEA-like protein in the range 400,000–500,000. Similar results, shown in Fig. 4, were obtained with the fractionated HT29 membrane preparation.

Figure 5 shows the results of blocking the binding of H58 with either CEA or HT29 membrane preparations which had previously been fractionated on the calibrated Sephadex G-200 column. In the case of CEA, heterogeneity of molecular size was evident with two partially resolved blocking bands at approximately 180,000–260,000. In some crude CEA preparations an additional band at approximately 400,000 was also observed. The fractionated HT29 membrane preparation also produced similar blocking of H58 by components of an approximate molecular weight of 200,000 but in addition molecular species with molecular weights of approximately 500,000 also blocked H58. Interestingly, our routine antiserum PKIG was not blocked by this very-high-molecular-weight protein but was blocked strongly by CEA of a more conventional size. SDS gel electrophoresis of purified CEA coupled with the Western blot procedure using radiolabelled immunopurified H58 was poorly resolved in the range $>20 \times 10^4$ daltons but showed H58 to bind to two distinct molecular entities present in the CEA preparation. In

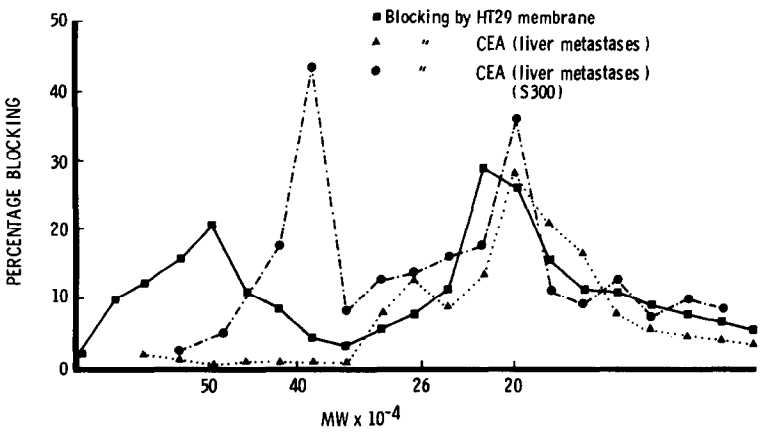

Fig. 5. Blocking of the binding of H58 to HT29 membrane preparation on target plate by Sephadex G-200 column fractions of HT29 membrane preparation and two preparations of CEA obtained from liver metastases.

contrast, PKIG in the same experiment showed a single but more diffuse band of lower molecular weight with the same CEA preparation.

Competitive blocking experiments

Between H58 and PKIG. The results of the blocking of H58 by our conventional goat polyclonal anti-CEA PKIG are shown in Fig. 6. Binding of H58 over the range 1:100–1:12,800 was not blocked by PKIG or by normal goat serum. As expected, including both PKIG and horse anti-goat Ig in the titration did produce constant high binding. Furthermore, the enhanced maximum binding of PKIG compared to H58 indicates that PKIG reacts with additional antigenic sites, probably on a different population of CEA.

The effect of H58 on the titration of PKIG is shown in Fig. 7. Again the absence of any inhibition of the binding of PKIG by H58 shows that the two antibodies react with different sites. However, in this case the lack of any increased binding indicates that essentially all the CEA expressing H58 binding sites also express PKIG binding sites. The presence of additional CEA binding PKIG was demonstrated in this experiment by the higher plateau value for the binding of PKIG compared to that of H58.

Between H58 and monoclonal antibody MA/1. This experiment was designed to show the effect on target binding by using a mixture of H58 with another monoclonal antibody, MA/1, known to have a different binding specificity. Saturating levels for H58 and MA/1 used individually were reached at concentrations of 12.5 and 25 μg/ml respectively (Fig. 8). Using the mixture of antibodies produced an increase in binding at dilutions below saturation. At higher concentrations of antibody, however, where all available binding sites should be occupied, the increase in binding produced by the mixture was smaller. Similar results were obtained with the HT29 membrane preparation and purified CEA targets. A non-specific IgG_1 control, W14, did not bind significantly to either target.

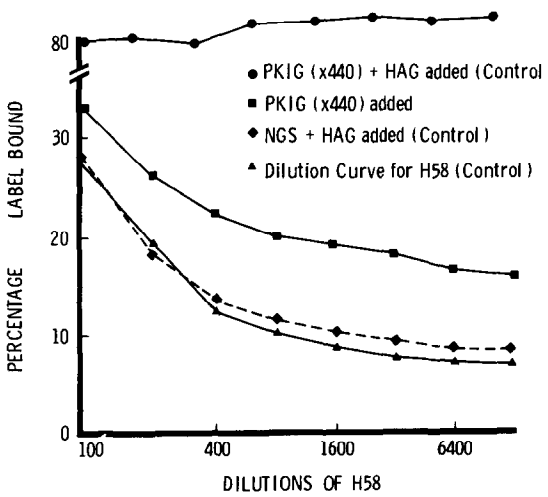

Fig. 6. Blocking of H58 by PKIG.

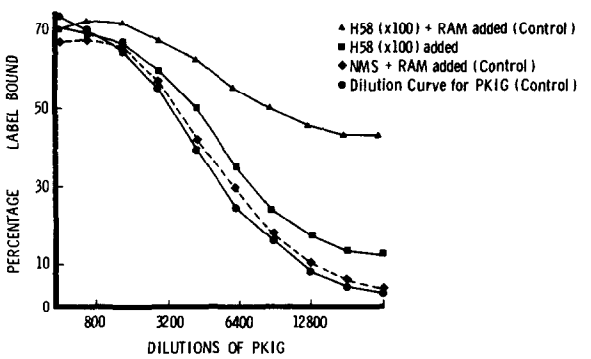

Fig. 7. Blocking of PKIG by H58.

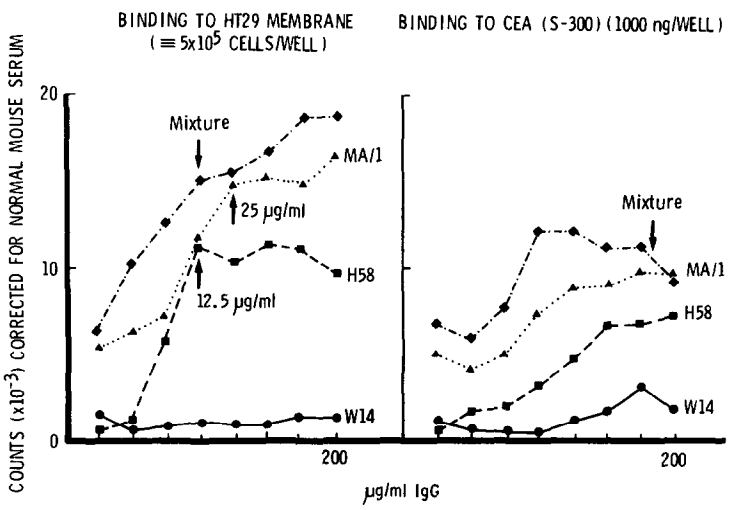

Fig. 8. Competitive binding between purified H58 and monoclonal antibody MA/1.

DISCUSSION

In this study we have chosen to use live HT29 tumour cells in an attempt to raise monoclonal antibodies against CEA. This should avoid the modification and partial destruction of this antigen known to occur on extraction [10, 11]. Some disruption of cell surfaces is to be expected on immunisation and again during trypsinisation used to obtain a viable cell suspension. However, conventionally prepared CEA is known to be resistant to trypsin in as much as its binding to polyclonal antibody is unaffected [12].

Our experience with the solid-phase assays using tumour extracts and a range of 'normal' controls has shown that considerable caution is necessary for interpretation of the results. Antibody H20, for instance, appeared promising on the initial screening but the specificity of this antibody for both 'normal' and neoplastic tissues was revealed only by the histological screening. Antibody H58, on the other hand, showed a good correlation between the results of the solid-phase assays and histology. In both cases the tumour tissues bound significantly more antibody than the 'normal' controls. The microtitre plate assay appeared to give meaningful binding data about H58 providing non-specific binding was corrected for using a non-immune immunoglobulin preparation identical to that of H58. Thus the protein A binding fraction of 'sham' ascitic fluid, and a non-specific IgG_1, proved adequate controls for the two purified preparations of H58.

It is likely that an antigen expressing cross-reactive binding sites with CEA represents a dominant tumour marker, expressed on HT29 cells. Almost a quarter of the HT29 binding antibodies initially selected reacted with purified CEA. The IgG subclass of H58 was determined using rabbit anti-mouse IgG_1 from two independent sources. However, its binding to, and elution from, the protein A–Sepharose differed from that reported for other mouse IgG_1 antibodies [13].

Unlike our routine polyclonal antiserum PKIG, H58 binds to, and is blocked by, HT29 membrane components of molecular weight 500,000, as well as CEA of more conventional size. This may be due to the fact that HT29 cells were used as the antigen. The existence of high-molecular-weight CEA has been shown previously by several workers using polyclonal antibodies [11, 14, 15]. The presence of large-sized CEA could be due to aggregation or the association of CEA with other protein. However, the failure of PKIG to react with the large-sized membrane component would necessitate masking of the PKIG binding sites but not the H58 binding sites, and this seems unlikely. Non-dissociable CEA of molecular weight 40×10^4 has previously been found in the serum of patients with cancer [16]. Other studies have also suggested that different immunological structures may be associated with CEA expressed on cell membranes compared to the conventionally prepared antigen [17].

Antisera able to bind preferentially to determinants on membrane CEA-like antigens may have a particular role in tumour imaging or drug targeting. H58 appears to have some of the properties required. It reacts with CEA from tumour cell and CEA secreted directly from the cell into culture medium. This property may be more important than high binding affinity for conventionally prepared CEA. It is likely that the binding kinetics or immunological expression of determinants appropriate to these different types of CEA will not be the same. It is of interest that H58 binds to a different binding site on CEA to that bound by our polyclonal PKIG serum currently used for tumour imaging in our laboratory. The competitive binding suggests that a main species of CEA exists able to bind both antibodies with the possibility of an additional species of CEA reacting with the polyclonal serum only. Similar conclusions on the intermolecular heterogeneity of CEA were drawn in our previous studies with monoclonal antibodies MA/1 and MA/200 [7].

That the two monoclonal antibodies MA/1 and H58 react with different binding sites was confirmed in the co-titration experiment. Using both antibodies together produced a significant increase in the amount of antibody bound per μg of IgG, providing the dilutions were such that available binding sites were only partially blocked. This condition would exist when radiolabelled antibodies are used *in vivo* and suggests that use of mixtures of antibodies for tumour imaging should be examined.

In conclusion, this study has demonstrated the production of a monoclonal antibody which reacts with an antigen of molecular size 50×10^4 expressed on HT29 colon tumour cells. The epitope with which H58 reacts is also expressed on CEA of conventional molecular size. This and our previous study [7] have shown that monoclonal antibodies react with their own unique binding sites on CEA and emphasise the need to explore immunological expression of CEA-like activity on colon tumour cells more fully.

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