Journal of Cellular Biochemistry 50:324–335 (1992)

Induction of an Immune Response Through the Idiotypic Network With Monoclonal Anti-Idiotype Antibodies in the Carcinoembryonic Antigen System

Jane Z. de Moraes, Célia R.W. Carneiro, Franz Buchegger, Jean-Pierre Mach, and J. Daniel Lopes

Cell Biology Department, Escola Paulista de Medicina, São Paulo, SP, 04023, Brazil (J.Z.d.M., J.D.L.); Ludwig Institute for Cancer Research (São Paulo Branch), São Paulo, SP, 01509, Brazil (C.R.W.C.); Biochemistry Institute, University of Lausanne, CH 1066 Épalinges, Switzerland (F.B., J.-P.M.)

Abstract Anti-idiotype antibodies can mimic the conformational epitopes of the original antigen and act as antigen substitutes for vaccination and/or serological purposes. To investigate this possibility concerning the tumor marker carcinoembryonic antigen (CEA), BALB/c mice were immunized with the previously described anti-CEA monoclonal antibody (MAb) 5.D11 (AB1). After cell fusion, 15 stable cloned cell lines secreting anti-Ids (AB2) were obtained. Selected MAbs gave various degrees of inhibition (up to 100%) of the binding of ^{125}I-labeled CEA to MAb 5.D11. Absence of reactivity of anti-Id MAbs with normal mouse IgG was first demonstrated by the fact that anti-Id MAbs were not absorbed by passage through a mouse IgG column, and second because they bound specifically to non-reduced MAb 5.D11 on Western blots. Anti-5.D11 MAbs did not inhibit binding to CEA of MAb 10.B9, another anti-CEA antibody obtained in the same fusion as 5.D11, or that of several anti-CEA MAbs reported in an international workshop, with the exception of two other anti-CEA MAbs, both directed against the GOLD IV epitope. When applied to an Id-anti-Id competitive radioimmunoassay, a sensitivity of 2 ng/ml of CEA was obtained, which is sufficient for monitoring circulating CEA in carcinoma patients. To verify that the anti-Id MAbs have the potential to be used as CEA vaccines, syngeneic BALB/c mice were immunized with these MAbs (AB2). Sera from immunized mice were demonstrated to contain AB3 antibodies recognizing the original antigen, CEA, both in enzyme immunoassay and by immunoperoxidase staining of human colon carcinoma. These results open the perspective of vaccination against colorectal carcinoma through the use of anti-idiotype antibodies as antigen substitutes.

Key words: colorectal carcinoma, CEA, idiotype, GOLD I–V, tumor vaccine

Carcinoembryonic antigen (CEA), first described by Gold and Freedman in 1965, is a highly glycosylated 180 kDa glycoprotein detectable in the blood of patients with various forms of carcinoma and which now has a widespread use as a tumor marker (Shiverly and Beatty, 1985; Chandrasekaran et al., 1983). High serum levels of CEA correlate with poor prognosis of colorectal carcinoma and the elevation of titers after surgical treatment is indicative of recurrence or tumor metastasis [for a review see Shively and Beatty (1985) and Jessup and Thomas (1989)]. Specific monoclonal antibodies (MAbs) were raised against the antigen and are replacing polyclonal antisera in quantitative assays (Buchegger et al., 1982; Carneiro et al., 1987). Radiolabeled MAbs are now also being employed for tumor diagnosis by immunoscintigraphy as well as for therapeutic purposes with various degrees of success (Goldenberg et al., 1978; Mach et al., 1980; Bischof-Delaloye et al., 1989; Buchegger et al., 1990; Sharkey et al., 1988). However, reproducibility of results from different groups is hampered for a number of reasons. CEA is part of a large family of proteins, some of which are expressed by normal granulocytes and macrophages and have been called non-specific cross-reacting antigens (Shively and Beatty, 1985; Buchegger et al., 1984; Thompson et al., 1989). Glycosylation of the molecule may not be uniform, leading to the

Abbreviations used: CEA, carcinoembryonic antigen; MAb, monoclonal antibody; Id, idiotype; MHC, major histocompatibility complex.

Received February 26, 1992; accepted July 14, 1992.

Address reprint requests to J. Daniel Lopes, Disciplina de Biologia Celular, Escola Paulista de Medicina, Rua Botucatu 862-8, São Paulo, SP, 04023, Brazil.

expression of different epitopes. Furthermore, CEA preparations may not be entirely pure or may not be identical from one laboratory to another (Monestier et al., 1989). MAbs produced against different CEA preparations, besides recognizing at least five of the different epitopes on the molecule (Hammarström et al., 1989), may exhibit different specificities and thus are not comparable among themselves (Haggarty et al., 1986; Hammarström et al., 1989). In addition, when MAbs are used for tumor detection or treatment of patients, since they are xenogeneic, they induce an immune response that further limits their use. Finally, CEA is not immunogenic in man, thus restricting its use for vaccination purposes.

An approach that could circumvent some of these difficulties would be the use of monoclonal anti-idiotype antibodies (anti-Id) directed against anti-CEA MAbs. Idiotypes (AB1) comprise the variable parts of the immunoglobulin molecule responsible for antigen recognition, and they themselves elicit an anti-Id response (AB2). These interactions could play an important role in the regulation of the immune response (Jerne, 1974). Some of the anti-Ids, named AB2, can conformationally mimic the original antigen and may be used as vaccines to induce AB3 antibodies that will recognize the antigen, an approach already being pursued for CEA (Bhattacharya-Chatterjee et al., 1990), other tumor markers in animal models, as well as for CO17-1A antigen (Herlyn et al., 1986, 1987a) and for HMW-MAA melanoma antigen (Mittelman et al., 1990), both in human patients. In the present work we describe the production and epitope characterization of syngeneic anti-Id MAbs raised in response to the previously described anti-CEA MAb 5.D11 (Carneiro et al., 1987). We also describe a competitive radioimmunoassay for measurement of serum CEA levels with Id-anti-Id MAbs. The possibility of vaccination against the antigen is demonstrated by the induction in syngeneic mice of an AB3 specific response to CEA through immunization with AB2 anti-idiotype antibodies.

MATERIALS AND METHODS

Monoclonal Anti-CEA Antibody

5.D11 and 10.B9 are previously described anti-CEA MAbs obtained in BALB/c mice. 5.D11, an IgG_{2a} MAb, is used as capture antibody in a quantitative CEA assay (Carneiro et al., 1987). 10.B9 is an IgG_1 MAb which recognizes a different epitope on the CEA molecule.

Immunization Protocol

To obtain AB2, 20 two-month old BALB/c female mice, divided into groups of four, were injected subcutaneously with 100 μg of the purified Mab 5.D11 (Carneiro et al., 1987) coupled to Keyhole-limpet hemocyanin by the glutaraldehyde method (Chow et al., 1985) and emulsified in complete Freund's adjuvant. Inoculation was in four different sites (inguinal and axillary) and the procedure was repeated after 15 days with the antigen now emulsified in incomplete Freund's adjuvant. The same amount of antigen was then injected intravenously every 15 days to a maximum of six injections, the last being performed 3 days before cell fusion. Before the last three immunizations, animals were bled and their sera tested for the presence of anti-Id antibodies against MAb 5.D11. To obtain AB3, BALB/c mice were immunized by the same protocol with the presently described anti-Id 6.C4 (AB2). The sera from the immunized mice were then tested for the presence of anti-Id antibodies against MAb 6.C4 or for antibodies reacting with CEA.

Production of MAbs

After the last booster inoculation, the mouse presenting the highest serum titer for anti-Id activity from the first immunized group, as assayed by an inhibition of binding of CEA to MAb 5.D11, was sacrificed. Cell fusion of its splenocytes with the non-secreting plasmacytoma SP2/0 was performed according to a previously described protocol (Lopes and Alves, 1983). Supernatants were tested by the same assay and the cells from positive wells were cloned by limiting dilution. Supernatants from positive clones were tested for antibody isotype characterization by the double-diffusion (Ouchterlony) method using specific antisera (Sigma Chemical Co., St. Louis, MO) for heavy and light mouse Ig chains.

Inhibition of Binding Assay

This assay was employed for testing both the immunized mouse serum and hybridoma supernatants. CEA was purified from liver metastasis of human colon carcinoma by perchloric acid extraction, followed by sequential Sephadex

G-200 and Sepharose 6B filtration (Krupey et al., 1985; Fritshe and Mach, 1977) and was labeled with ^{125}I by the IodoGen method (Fraker and Speck, 1978) to an average specific activity of 3.5 μCi/μg. Fifty microliters of a solution containing 10 μg/ml (w/v) of capture antibody (MAb 5.D11, unless otherwise stated) in phosphate buffered saline (PBS; 150 mM NaCl, 10 mM phosphate, pH 7.2) were applied to each well to coat 96-well polyvinyl plates (Falcon, Oxnard, CA). Blocking of the remaining active sites on plastic was made with 200 μl of 1% bovine serum albumin (BSA, Sigma) in PBS (PBS-BSA).

Hybridoma supernatants (50 μl/well) were added to the coated wells and, after 1 h of incubation at 37°C, plates were washed with PBS-BSA. Labeled CEA (200,000 cpm/well) was then added and, after 1 h incubation and thorough washing, the wells were cut and counted in an LKB MiniGamma counter (LKB, Bromma, Sweden). Supernatants of SP2/0 cell cultures, complete culture medium, and normal mouse serum diluted 1:100 in PBS were used as controls.

Reverse direct and indirect assays, with CEA bound to polystyrene plastic balls as the solid phase (Precision Plastic Ball Co., Chicago, Ill.), were devised to determine idiotype recognition against a previously described panel of anti-CEA MAbs obtained from different laboratories. This panel of MAbs allowed identification of at least five different epitopes on the CEA molecule called GOLD I to V (Hammarström et al., 1989). In the direct assay, anti-Id MAb 6.C4 was pre-incubated with each of the different ^{125}I-labeled anti-CEA MAbs for 2 h at 37°C before the addition of CEA coated plastic balls. In the indirect assay, unlabeled 6.C4 was mixed with each unlabeled anti-CEA MAb, the coated balls were added, and the reaction evaluated by the addition of ^{125}I-labeled $F(ab')_2$ rabbit anti-mouse IgG. In both cases, balls were finally washed and counted for bound iodine. The different anti-CEA MAbs or the anti-mouse IgG $F(ab')_2$ Id MAbs were labeled with ^{125}I by the Iodogen Method (Fraker and Speck, 1978) with an average specific activity of 10 μCi/μg.

Production of $F(ab')_2$ Fragments

MAb 5.D11 was digested for 4 h with 3% pepsin (Sigma) in 100 mM acetate buffer, pH 4.2, at 37°C, for the production of $F(ab')_2$ fragments, as described (Hudson and Hay, 1976). After purification by Sephadex G-150 filtration, the $F(ab')_2$ fragments obtained were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE).

SDS-PAGE and Western Blotting

This was performed on vertical 10% acrylamide slab gels, under reducing and non-reducing conditions, according to Laemmli (1970). MAb 5.D11 Western blots were performed as already described (Lopes et al., 1985). Reducing conditions, obtained through the addition of 2-mercaptoethanol, were used for the detection of the antibody separate chains, on 10% acrylamide gels. Non-reducing conditions, in which reducing reagents were omitted, were used for the detection of whole antibody on 5% acrylamide gels.

Affinity Chromatography

Purified mouse IgG was obtained by affinity chromatography on a Protein A-Sepharose 4B column (Pharmacia Fine Chemicals, Uppsala, Sweden) and was coupled to CNBr-activated Sepharose (Pharmacia) according to the manufacturer's instructions. Anti-Id MAbs were tested for their ability to recognize 5.D11 $F(ab')_2$ fragment before and after being chromatographed through the adsorbent column.

Quantitative Assays for CEA

Samples with known concentrations of CEA for the standard curve were prepared by either dissolving purified CEA in PBS-0.1% BSA or by using human serum samples in which the CEA levels had been previously measured using commercial kits. The competition assays consisted of a MAb coating the solid phase, either 5.D11 or an anti-Id MAb, 50 μl/well of a solution of 10 μg/ml of antibody in PBS, and a ^{125}I-labeled second antibody, used to compete with CEA present in the test samples. The final volume of the mixture of labeled antibody and CEA samples was always 50 μl. The second antibody was either an anti-Id, when 5.D11 was on the solid phase, or an anti-CEA MAb, when anti-Id was on the solid phase. Anti-CEA MAbs used were 5.D11 or 202 (Buchegger et al., 1982). Labeled antibody was added to samples containing different amounts of CEA and the mixtures were distributed in wells previously coated with anti-

body and quenched with PBS-BSA. Reactions were incubated for 2 h under gentle agitation at 37°C. When the labeled antibody was MAb 5.D11, before addition to the wells, a previous overnight incubation at 37°C of the mixture was made. Wells were then thoroughly washed, cut, and counted for bound iodine in an LKB Mini-Gamma counter.

Production and Characterization of AB3 Antibodies

After immunization with AB2 antibodies, as described for immunization with AB1, BALB/c mice were bled and their sera tested for the recognition of CEA through both enzyme-immunoassay and immunoperoxidase staining of frozen sections from human colon carcinoma. Enzyme-immunoassay was performed with CEA (0.5 μg/ml in PBS, pH 7.5) bound to the solid phase, as described (Carneiro et al., 1987). Briefly, 50 μl of serum, serially diluted in PBS-BSA from 1:80 to 1:2560, was added to CEA coated and BSA quenched wells on polyvinyl plates. After 2 h of incubation at 37°C and three washes with PBS-BSA, biotinylated anti-mouse IgG were added, followed by streptavidin-peroxidase conjugate (Sigma). Reactions were developed with ortho-phenylenediamine in 0.1 M acetate phosphate buffer, pH 5.8, containing 0.03% H_2O_2, and read in a Titertek EIA Reader. Inhibition of binding of AB3 to CEA was performed by previous overnight incubation at 37°C, of mouse no. 2 serum, diluted 1:320 in PBS-BSA, with serial dilutions of purified MAb 6.C4. An unrelated MAb of the same IgG2b isotype was used as control. Direct binding of AB3 sera, diluted from 1:10 to 1:160 in 0.04 M Tris, pH 7.5, containing 0.01% BSA, to ^{125}I-labeled CEA was measured through ammonium sulfate precipitation at the final concentration of 50%. The pellet was counted in a LKB MiniGamma Counter.

Immunoperoxidase staining of tissue sections of human CRC by AB3 was performed with biotin-avidin reagents (Vector, Burlingham, CA) as described (Guesdon et al., 1979). Endogenous peroxidase was blocked with methanol containing 3% H_2O_2. Staining was made with 3-amino-9-ethyl-carbazole dissolved in 2.5 ml of dimethylformamide and H_2O_2 0.015% in 50 ml of 0.05 M acetate buffer, pH 5.2 (Burtin et al., 1979). All proper controls for specificity, such as omission of the first incubation step, were performed. Normal mouse serum, at the same serial dilutions used for positive sera, was employed as a negative control.

TABLE 1. Inhibition of Binding of CEA to MAb 5D11 by 15 Anti-Id MAbs of Different Isotypes

Anti-Id MAbs	Isotype	% Binding inhibition	
		^{126}I-CEA[a]	^{126}I-5.D11[b]
1.B7[c]	IgG1,k	99.8	100.0
2.B11	—	99.3	98.4
3.B11	—	99.8	98.8
4.E3[c]	IgG1,k	99.9	98.1
4.F6[c]	IgG1,k	99.8	97.0
5.A3	—	29.5	17.3
5.C4	IgG1,k	98.8	98.4
6.C4[c]	IgG2b,k	100.0	100.0
6.C5[c]	—	61.1	34.8
6.F7	—	22.8	14.2
7.H6[c]	IgG1,k	27.9	21.7
8.C9[c]	IgG2b,k	67.5	30.7
8.G11	IgG1,k	99.8	98.1
9.B9	IgG2b,k	23.2	0.0
9.E10[c]	IgG2b,k	88.4	74.9

[a]Percentages of inhibition of binding of ^{126}I-CEA to MAb 5D11 linked to solid phase, in excess of different anti-Id MAbs.
[b]Percentages of inhibition of binding of ^{126}I-MAb 5D11 to solid phase CEA in excess of different anti-Id MAbs.
[c]Selected clones for further evaluation.

RESULTS

Anti-Idiotype MAbs

Uninhibited binding of ^{125}I-labeled CEA to MAb 5.D11 was roughly around 20%. Fifteen clones whose supernatants showed inhibition of binding of labeled CEA to MAb 5.D11 were established as stable cell lines. Ten of them had their isotypes characterized: six IgG1 and four IgG2b, all "kappa" light chains. Levels of inhibition obtained with undiluted supernatants ranged from 23 to 100% (Table I). Comparable inhibition levels were also obtained when the supernatants were preincubated with labeled 5.D11 and then added to the solid phase coated with purified CEA, except for anti-Id MAb 10.B9, which did not inhibit the binding of ^{125}I-labeled 5.D11 to solid phase CEA.

In Western blots against reduced and non-reduced 5.D11, positive reactions with biotinylated anti-Id MAbs were observed only with non-reduced 5.D11 as shown in Figure 1, a composition of two different experiments: whole, non-reduced antibody was run on 5% acrylamide (lanes A and B), and reduced, separated

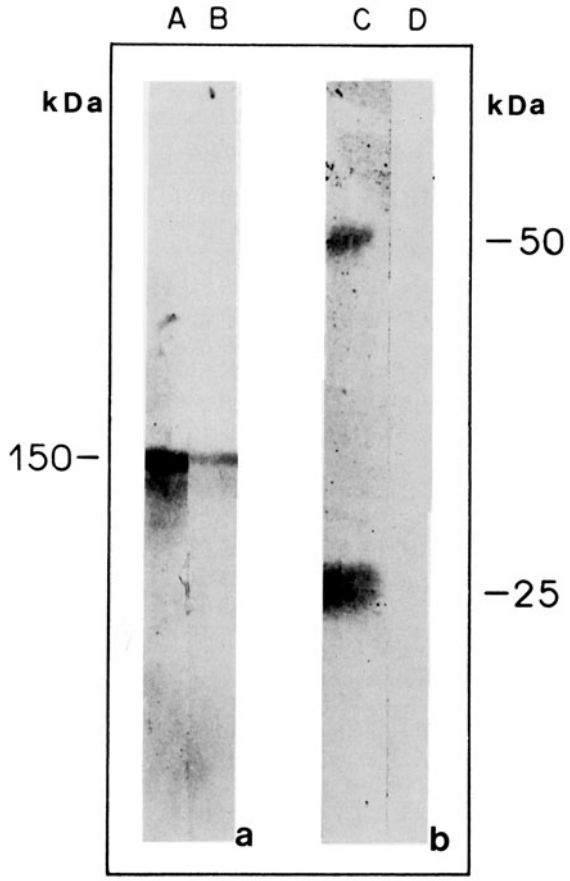

Fig. 1. Western blots of non-reduced (a) and reduced (b) 5.D11, developed with either peroxidase-conjugated anti-mouse IgG (lanes A and C) or biotinylated MAb 6.C4 followed by streptavidin-peroxidase (lanes B and D). Lanes A and B were run on 5% acrylamide while lanes C and D were run on 10% acrylamide gels. The figure is a composition of two different experiments, thus producing nitrocellulose strips of different shapes. Side numbers represent molecular mass of the detected bands. Anti-Id MAb 6.C4 recognizes 5.D11 only under non-reducing conditions.

antibody chains were run on 10% acrylamide (lanes C and D). Since this assay involved two murine antibodies, a direct reaction was mandatory, with a labeled second antibody. For this reason, only the pre-selected MAbs 6.C4 and 9.E10 were biotinylated and tested, with similar results.

Specificity of Anti-Id MAbs

Two anti-CEA MAbs were used for the screening of anti-Id MAbs: 5.D11, which was the MAb used for the immunization of mice, and 10.B9, another anti-CEA MAb obtained in the same fusion experiment, which is known to recognize a different epitope on the CEA molecule (Carneiro et al., 1987). Labeled anti-Id 8.C9 MAb

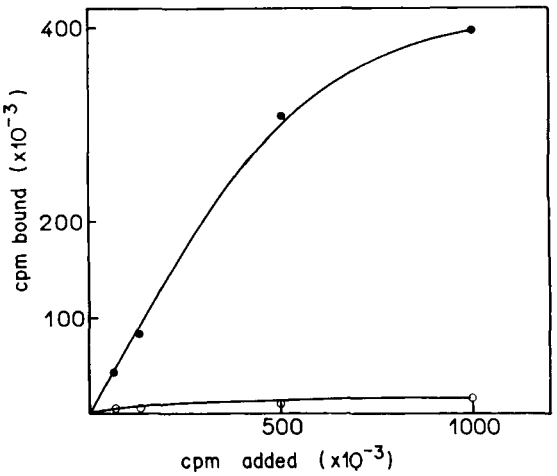

Fig. 2. Binding of anti-Id MAb 6.C4 to F(ab')2 fragment of MAb 5.D11 (●—●) and to an unrelated antibody (○—○).

bound to 5.D11 MAb in solid phase but not to 10.B9 or to an unrelated MAb anti-*S. mansoni*. Similar results were observed for anti-Id MAb 1.B7, 6.C4, and 9.E10 (data not shown). Also, when anti-Id MAbs were previously passed through a normal mouse IgG-Sepharose column and then retested for binding to solid phase MAb 5.D11, the binding was unchanged. Binding of 6.C4 MAb to solid phase was also unchanged when $F(ab')_2$ fragments of MAb 5.D11 instead of the whole antibody were used to coat the plastic balls (Fig. 2).

Epitope Characterization

When tested against a panel of anti-CEA MAbs, classified as GOLD I to GOLD V according to the epitopes recognized in the CEA molecule (Hammarström et al., 1989), the pre-selected MAb 6.C4 inhibited the binding to CEA of some GOLD IV MAbs, but not all, suggesting that there are several subspecificities within the group of anti-GOLD IV MAbs. None of the MAbs specific for the other CEA epitopes were inhibited either by a direct or an indirect inhibition assay (Fig. 3). The other AB2 MAbs were not tested against the panel. From those tested, only MAbs 202 and CE.25, both classified as GOLD IV, were convincingly inhibitory. These results strongly suggest that the original AB1 (MAb 5.D11) was also a GOLD IV MAb. To confirm this assumption, some representative GOLD MAbs (B.93, GOLD I; 35, GOLD II; B.7, GOLD III; CE 25 and 202, both GOLD IV; and 192, GOLD V) were tested for their ability to inhibit

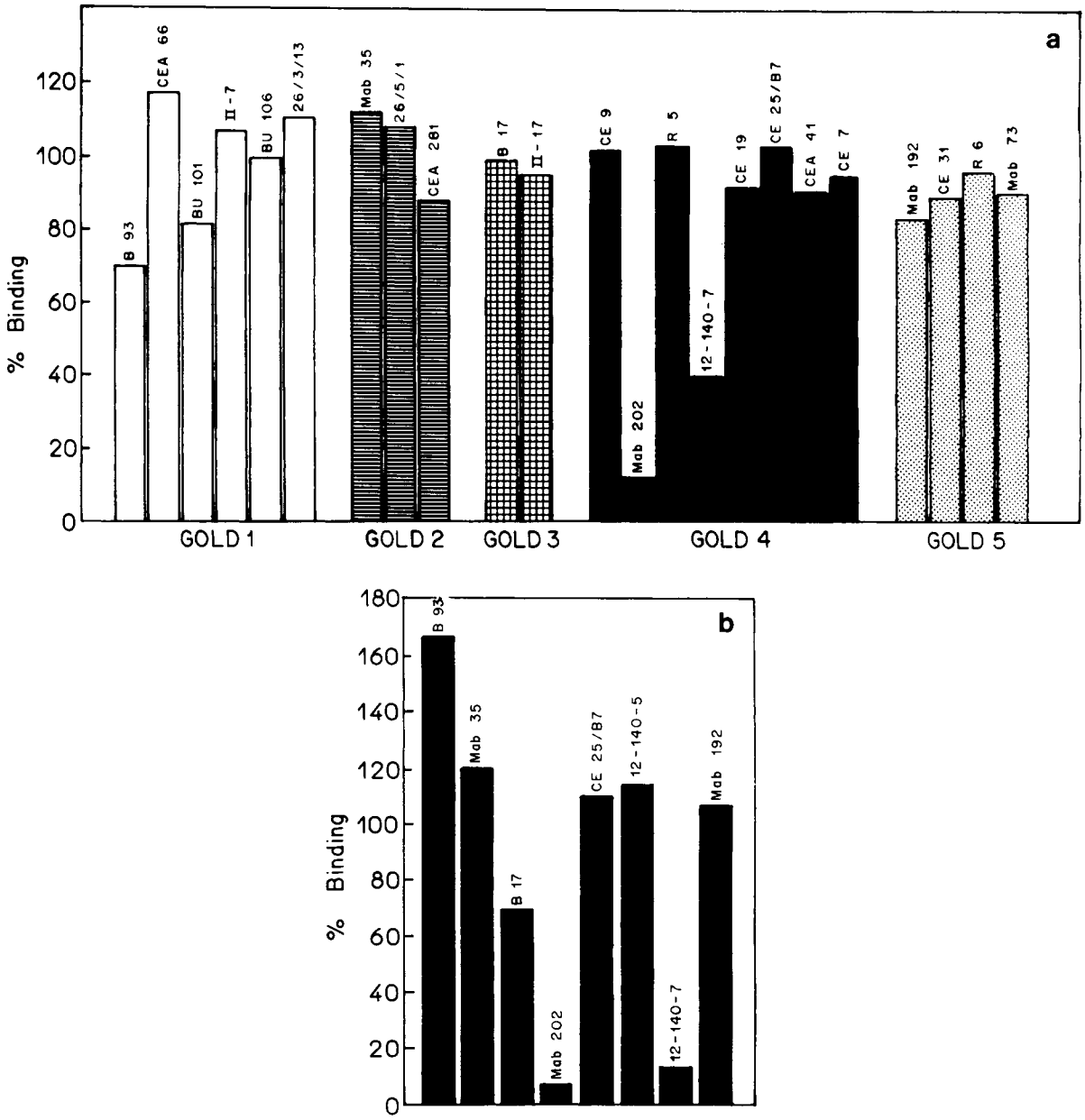

Fig. 3. Inhibition of binding of GOLD-classified MAbs to CEA immobilized on solid phase by anti-Id MAb 6.C4 using indirect (**a**) and direct (**b**) assays. Results are expressed as % of the binding without unlabeled MAb 6.C4. Important inhibition was observed only against two GOLD IV MAbs (202 and 12-140-7).

binding of ^{125}I-labeled MAb 5.D11 to Sepharose-bound CEA (data not shown).

Quantitative Assay for Measuring CEA Levels

Figure 4 shows a typical standard curve for quantitation of CEA. In this case, 5.D11 was bound to the solid phase and labeled ^{125}I-labeled 6.C4 was used as antibody in competition with the CEA containing sample. Similar results were obtained when 6.C4 was bound to the solid phase and either ^{125}I-labeled MAb 5.D11 or 202 were used as antibodies in competition with CEA (data not shown).

Characterization of the AB3 Response

Sera from mice immunized with 6.C4 bound to CEA present on the solid phase as demonstrated by enzyme immunoassay (Figure 5a). Direct binding of labeled CEA by AB3 serum (mouse no. 2 serum) confirmed the EIA results

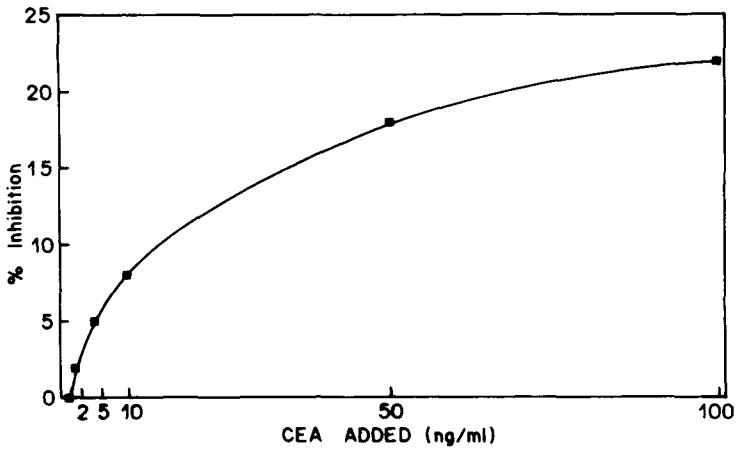

Fig. 4. Competitive radioimmunoassay for the measurement of CEA levels with labeled anti-Id MAb 8.C9 and MAb 5.D11 bound to the solid phase. CEA levels as low as 2 ng/ml could be discriminated.

(data not shown). Binding of one of those sera to CEA (mouse serum no. 2, which gave the highest titer against the antigen) was inhibited by preincubation with MAb 6.C4 in a dose-dependent fashion, as shown in Figure 6B. The same serum recognized the antigen expressed on a human colon carcinoma, as observed by immunoperoxidase staining. MAb 5.D11 was used as a positive control and the normal mouse serum as negative control (Figs. 6A and B).

DISCUSSION

The striking characteristic of anti-idiotype antibodies is their potential ability to mimic, and therefore substitute, the original antigen in some of its biological and/or serological activities. It is therefore necessary that this antigen surrogate exhibit the internal image of the specific epitope. This holds true when anti-Ids are produced for use as vaccines to induce a specific immune response against a non-immunogenic antigen or against antigens which pose difficulties for large scale production (Monestier et al., 1989). No indirect test, however, can prove that an anti-Id is actually the internal image of the antigen. Such a conclusion can be drawn only after immunization procedures, when a specific immune response against the original antigen is detected.

The possibility of production of synthetic vaccines with anti-Ids has been already addressed using several models, mostly animal, as has been shown for *Schistosoma mansoni* (Grzych et al., 1985), *Trypanosoma rhodesiense* (Sacks and Sher, 1983), and *Plasmodium berghei* (Potoncjak et al., 1982), as well as bacteria (McNamara et al., 1984) and viral antigens (Kennedy et al., 1984). Tumor models have also been studied with some success and even a vaccination trial in humans with colorectal carcinoma was performed (Herlyn et al., 1987a,b). The efficacy of this approach, however, remains to be established.

No such restrictions apply when anti-Ids are used for serological purposes, where complete inhibition of the antigen binding to its original antibody is sufficient for the anti-Id to act as a reliable antigen substitute. This inhibition can be observed even when the anti-Id is not definitely characterized as an internal image to any of the known antigen epitopes. Moreover, an assay employing a MAb and its respective anti-Id would rely on two fully reproducible reagents, thus avoiding the pitfalls of antigen purification from different sources by different laboratories with dissimilar methods.

In this report, we describe the production of syngeneic anti-Id MAbs to the murine anti-CEA MAb 5.D11 that fulfill these requirements. The 5.D11 MAb was selected for the immunization since it is an already described anti-CEA MAb which reacted very well as capture antibody when bound to a solid phase in a two-site quantitative assay (Carneiro et al., 1987). All the 15 selected hybridoma anti-Id cell lines secrete antibodies that inhibit binding of CEA to 5.D11, from 23 to 100%. To demonstrate that none of the obtained MAbs had any anti-mouse IgG activity, anti-Ids were absorbed on a normal mouse IgG-Sepharose column, none of them

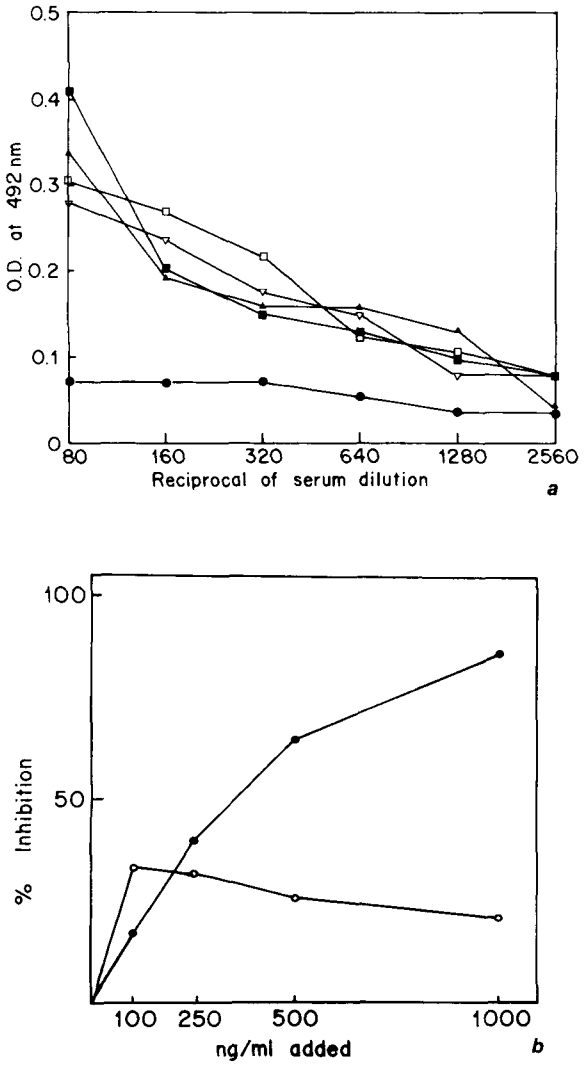

Fig. 5. a: Titration of AB2-immunized mouse sera against CEA bound to the solid phase by enzymeimmunoassay. Results with sera from four mice are presented with different symbols (□, ▲, ■, ▽). ●—●, control normal mouse serum. b: Inhibition of binding of mouse no. 2 serum (1:320) to CEA by increasing concentrations of purified MAb 6.C4 (●—●); unrelated antibody (○—○).

losing their inhibition ability. Also, binding of ^{125}I-labeled anti-Ids was similar to both the original 5.D11 antibody and its F(ab')$_2$ fragments. Anti-Id MAb 6.C4 was selected for further studies on the basis of its inhibition capacity of (100%). Furthermore, this specific MAb, showed to be the more stable after labeling with both ^{125}Iodine and biotin. MAb 6.C4, did not recognize isolated light and heavy chains of MAb 5.D11 in Western blots under reducing conditions, but did so under non-reducing conditions, suggesting that it reacts against a conformational idiotypic epitope shared by both antibody chains.

Specificity of the anti-Id MAbs was proven by two different approaches. First, anti-Ids did not bind to 10.B9, another anti-CEA MAb obtained in the same fusion experiment that originated 5.D11, but this is known to recognize a different epitope on the CEA molecule. Second, when tested against a panel of MAbs of known epitope specificity, only the MAbs known to react with GOLD IV epitope were inhibited in their binding to CEA by our anti-Id MAbs. Furthermore, when tested against a panel of eight anti-GOLD IV epitope antibodies, only two of these MAbs were inhibited by our anti-Id Mabs. MAbs recognizing the five different GOLD epitopes were also tested for their ability to inhibit binding of ^{125}I-labeled 5.D11 to CEA, and significant inhibition was only with MAbs 202 and CE.25, both classified as GOLD IV antibodies. These results indicate that 5.D11 is actually a GOLD IV MAb. On the other hand, the fact that only two of eight MAbs classified as recognizing the same GOLD IV CEA epitope are recognized by our anti-ID MAb shows that different antibodies can recognize the same epitope, without sharing the same idiotype. Therefore, anti-Ids can help to sort out different idiotypes among supposedly similar antibodies classified according to their reaction with the same epitope. This may be very important since the classification of epitope reactivity is made by competitive binding inhibition. This test when positive indicates that the two antibodies are reacting either with the same epitope or with a topographically close epitope which renders the other unaccessible by steric hindrance, after binding of the first antibody.

Quantitative assays for CEA were developed with anti-Id MAb 6.C4 and both anti-CEA MAbs 5.D11, against which the anti-Id MAb was raised, and 202, which was known to react with a GOLD IV epitope (Buchegger et al., 1984; Hammarström et al., 1989). Anti-Id was shown to react well either as a capture antibody bound to the solid phase or as a competing antibody in the soluble phase. Sensitivity of these assays seems good enough for clinical use, since CEA levels as low as 2 ng/ml could be discriminated. Such tests, by using two defined MAbs, do not depend on antigen purification. Therefore, they may also be useful for the quality control of CEA

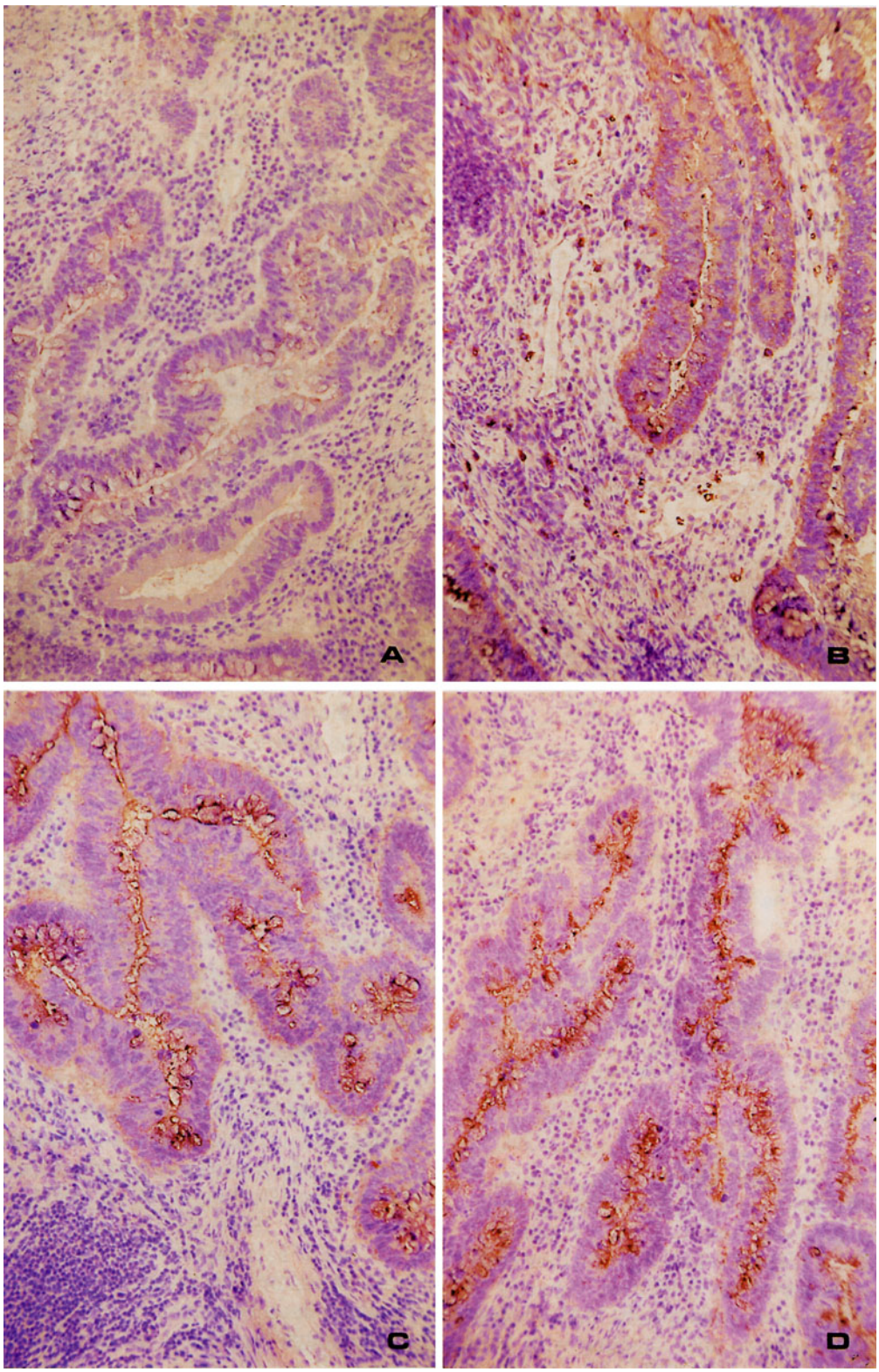

Fig. 6. Immunoperoxidase staining of tissue sections of human colon carcinoma by (**A**) normal mouse serum, (**B**) anti-CEA MAb 5.D11, originally use to induce AB2 antibodies, (**C**) anti-CEA MAb 35 A7, used as positive control and (**D**) AB3 serum (mouse no. 2 serum). It is interesting to notice that while MAb 5.D11 stains granulocytes, thus recognizing NCA, AB3 serum does not.

quantitative assays routinely used for the evaluation of cancer patients.

The ultimate application of anti-Ids would be the immunization of patients for the induction of an immune response against a nonimmunogenic antigen such as CEA. Experiments performed by several groups have shown that anti-CEA MAbs, radiolabeled with beta-emitting isotopes, have the potential to destroy colorectal carcinoma with a certain degree of specificity (Buchegger et al., 1990; Sharkey et al., 1988). Therefore, one may assume that a natural immune response could have the same capacity. Anti-Id antibodies seem to be able to activate silent clones (Bona et al., 1981; Schick et al., 1987) and break tolerance (Stein and Soederstroem, 1984) to the original antigen. The anti-Ids described here seem to fulfill most of the requirements for this type of study. To check this possibility, MAb 6.C4 was injected in mice and a detectable AB3 response was obtained, as measured by its specific binding to the original antigen, either purified and bound to the solid phase, or expressed by human CRC in histological sections. Also, AB2 6.C4 MAb blocked the binding of mouse AB3 sera to CEA, further confirming AB3 specificity. Our data are very similar to those described by Bhattacharya-Chatterjee et al. (1990). Interestingly however, their AB3 MAb did not recognize secreted CEA and it is hypothesized that this is dependent on the different immunogens (original Ag vs. its internal image).

Several arguments can be raised in favor or against the clinical use of anti-Id MAbs as tumor vaccines. Induction of cytolytic T cells to cells expressing the tumor antigen may be essential for the success of the vaccination procedure, as has already been suggested for different animal systems (Raychaudhuri et al., 1987a,b,c). This type of response is, however, restricted to syngeneic targets (Herlyn et al., 1987b), thus precluding experimental studies. On the other hand, injection of murine MAbs into patients will certainly elicit an immune response likely to recognize other segments of the anti-Id and therefore limiting the use of murine MAbs for any other clinical applications (Bhatacharya-Chatterjee et al., 1990). This could be minimized by the administration of mouse Fab fragments or human MAbs anti-murine Id (Austin et al., 1991). Whether an efficient T cell response will be produced in humans remains to be established.

Also, anti-Id immune response is genetically/restricted (Sacks and Sher, 1983), and a range of T cell epitopes will be presented by different sets of MHC haplotypes. This genetic limitation of the immune response could be circumvented by the use of several anti-Id MAbs directed against antibodies recognizing different epitopes of the same antigen or against different antigens of the same tumor. Notwithstanding all these possible limitations, which will certainly require further research, our results open the possibility of anti-Id vaccination of patients bearing CEA expressing tumors.

ACKNOWLEDGMENTS

We are indebted to R.R. Brentani for his critical comments and encouragement, to Roger Chammas and Magali Schreyer for the immunoperoxidase reactions, to Lionel Conrado B. de Souza for the scientific documentation, and to Edilberto Postol for technical assistance. J.Z.d.M. is a recipient of a grant from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). This work was supported by the Projeto de Apoio ao Desenvolvimento à Ciência e Tecnologia (PADCT-FINEP) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP).

REFERENCES

Austin EB, Robins RA, Baldwin RW, Durrant LG (1991): Induction of delayed hypersensitivity to human tumor cells with a human monoclonal anti-idiotypic antibody. J Natl Cancer Inst 83:1245–1248.

Bhattacharya-Chatterjee M, Mukerjee S, Biddle W, Foon KA, Kohler H (1990): Murine monoclonal antibody as a potential network antigen for human carcinoembryonic antigen. J Immunol 145:2578–2765.

Bischof-Delaloye A, Delaloye B, Buchegger F, Gilgien W, Studer A, Curchod S, Givel J-C, Mosimann F, Pettavel J, Mach J-P (1989): Clinical value of immunoscintigraphy in colorectal carcinoma patients: A prospective study. J Nucl Med 30:1646–1656.

Bona CA, Herbert-Katz E, Paul WE (1981): Idiotype-anti-idiotype regulation. I-Immunization with a levan-binding myeloma protein leads to the appearance of auto-anti-(anti-idiotype) antibodies and to the activation of silent clones. J Exp Med 153:951–967.

Buchegger F, Han M, Rivier D, Carrel S, Accola RS, Mach J-P (1982): Monoclonal antibodies against cancinoembryonic antigen (CEA) used in a solid-phase enzyme immunoassay. First clinical results. J Immunol Meth 49:129–139.

Buchegger F, Schreyer M, Carrel S, Mach J-P (1984): Monoclonal antibodies identify a CEA crossreacting antigen of 95kD (NCA-95) distinct in antigenicity and tissue distribution from previously described NCA of 55kD. Int J Cancer 33:643–649.

Buchegger F, Pelegrin A, Delaloye B, Bischof-Delaloye A, Mach J-P (1990): ^{131}Iodine-labeled MAb F(Ab')$_2$ fragments are more efficient and less toxic than intact anti-CEA antibodies in radioimmunotherapy of large human colon carcinoma grafted in nude mice. J Nucl Med 31:1035–1044.

Burtin P, Calmettes C, Fondaneche MC (1979): CEA and non-specific cross-reacting antigen (NCA) in medullary carcinomas of the thyroid. Int J Cancer 23:741–745.

Carneiro CRW, Lopes JD, Brentani MM (1987): Carcinoembryonic antigen (CEA): Production of immunoprecipitating monoclonal antibodies and development of an enzyme immunoassay. Hybridoma 6:689–692.

Chandrasekaran EV, Davila M, Nixon DW, Goldfarb M, Mendicino J (1983): Isolation and structures of the oligosacharide units of carcinoembryonic antigen. J Biol Chem 258:7213–7222.

Chow M, Yabrov R, Bittle J, Hogle J, Baltimore D (1985): Synthetic peptides from four separate of polyovirus type I capside protein VP1 induce neutralizing antibodies. Prc Natl Acad Sci USA 82:910–914.

Fraker PJ, Speck JC (1978): Protein and cell membrane iodinations with sparingly soluble chloroamide-1,3,4,6-tetrachloro 3a,6a-diphenylglicoluril. Biochem Biophys Res Commun 80:849–857.

Fritshe R, Mach J-P (1977): Isolation and characterization of carcinoembryonic antigen (CEA) extracted from normal human colon mucosa. Immunochemistry 14:119–127.

Gold P, Freedman SO (1965): Demonstration of tumor specific antigens in human colonic carcinomata by immunological tolerance and absorption techniques. J Exp Med 121:439–462.

Goldenberg DM, Deland F, Kim EE, Benett S, Primus FJ, van Nagell JR, Estes N, De Simone P, Rayburn P (1978): Use of radiolabeled antibodies to carcinoembryonic antigen for the detection and localization of diverse cancers by external photoscanning. N Engl J Med 298:1384–1388.

Grzych JM, Capron M, Lambert PH, Dissous C, Torres S, Capron A (1985): An anti-idiotype vaccine against experimental schistosomiasis. Nature 316:74–76.

Guesdon J-L, Ternynk T, Avrameas S (1979): The use of avidin-biotin interaction in immunoenzymatic techniques. J Histochem Cytochem 27:1131–1139.

Haggarty A, Legler C, Krantz MJ, Fuks A (1986): Epitopes of carcinoembryonic antigen defined by monoclonal antibodies prepared from mice immunized with purified carcinoembryonic antigen or HCT-8R cells. Cancer Res 46:300–309.

Hammarström S, Shively JE, Paxton RJ, Beatty BG, Larsson A, Gosh R, Bormer O, Buchegger F, Mach J-P, Burtin P, Seguin P, Darbouret B, Degorce F, Sertour J, Jolu JP, Fuks A, Kalthoff H, Schmiegel W, Ardnt R, Kloppel S, Grunert F, Schwarz K, Matsuoka Y, Kuroki M, Wagener C, Weber T, Yachi A, Imai K, Hishikawa N, Tsujisaki M (1989): Antigenic sites in carcinoembryonic antigen. Cancer Res 49:4852–4858.

Herlyn D, Sears HF, Iliopoulos D, Lubeck M, Douillard J-Y, Sindelar W, Tempero M, Mellstedt H, Maher M, Koprowski H (1986): Anti-idiotypic antibodies to monoclonal antibody CO17-1A. Hybridoma 5 (Suppl. 1):S51–58.

Herlyn D, Ross AH, Iliopoulos D, Koprowski H (1987a): Induction of specific immunity to human colon carcinoma by anti-idiotypic antibodies to monoclonal antibodies CO17-1A. Eur J Immunol 17:1649–1652.

Herlyn D, Wettendorf M, Schmoll E, Iliopoulos D, Schedel I, Dreikhausen U, Raab R, Ross AH, Jaksche H, Scriba M, Koprowski H (1987b): Anti-idiotype immunization of cancer patients: Modulation of the immune response. Proc Natl Acad Sci USA 84:8055–8059.

Hudson L, Hay FC (1976): Cleavage of IgG by proteolytic enzymes. In: "Practical Immunology." London: Blackwell Scientific Publications, pp 186– 188.

Jerne NK (1974): Towards a network theory of the immune system. Ann Immunol (Paris) 125C:373–389.

Jessup JM, Thomas P (1989): Carcinoembryonic antigen: function in metastasis by human colorectal carcinoma. Cancer Metastasis Rev 8:263–280.

Kennedy RC, Melnyck JL, Dreesman GR (1984): Antibody to hepatitis B virus induced by injecting antibodies to the idiotype. Science 223:930–931.

Krupey J, Gold P, Freedman SO (1985): Physicochemical studies of the carcinoembryonic antigens of the human digestive system. J Exp Med 128:387–398.

Laemmli UK (1970): Cleavage of structural proteins during the assembly of head of bacteriophage T4. Nature 227:680–685.

Lopes JD, Alves MJM (1983): Production of monoclonal antibodies by somatic cell hybridization. In Morel CM (ed): "Genes and Antigens of Parasites." Rio de Janeiro: UNDP/WORLD BANK/WHO/FINEP/CNPq/FIOCRUZ, pp 386–400.

Lopes JD, dos Reis M, Brentani RR (1985): Presence of laminin receptors in *Staphylococcus aureus*. Science 229: 275–277.

Mach J-P, Carrel S, Forni M, Ritschard J, Donath A, Alberto P (1980): Tumor localization of radiolabeled antibodies against carcinoembryonic antigen in patients with carcinoma. N Engl J Med 303:5–10.

McNamara NK, Ward RE, Kohler E (1984): Monoclonal idiotope vaccine against *Streptococcus pneumoniae* infection. Science 226:1325–1326.

Mittelman A, Chen ZJ, Kageshita T, Yang H, Yamada M, Baskind P, Goldberg N, Puccio C, Ahmed T, Arlin Z, Ferrone S (1990): Active specific immunotherapy in patients with melanoma. J Clin Invest 86:2136–2144.

Monestier M, Debbas ME, Goldenberg DM (1989): Syngeneic anti-idiotype monoclonal antibodies to murine anti-carcinoembryonic antigen monoclonal antibodies. Cancer Res 49:123–126.

Potoncjak P, Zavala F, Nussenzweig R, Nussenzweig V (1982): Inhibition of idiotype-anti-idiotype interaction for detection of a parasite antigen: a new immunoassay. Science 215:1637–1639.

Raychaudhuri S, Saeki Y, Chen JJ, Iribe H, Fuji H, Kohler H (1987a): Tumor-specific idiotype vaccines. II. Analysis of the tumor-related network response induced by the tumor and by internal image antigens (Ab2§). J Immunol 139: 271–278.

Raychaudhuri S, Saeki Y, Chen JJ, Kohler H (1987b): Tumor-specific idiotype vaccines. III. Induction of T helper cells by anti-idiotypic and tumor cells. J Immunol 139: 2096–2102.

Raychaudhuri S, Saeki Y, Fuji H, Kohler H (1987c): Tumor-specific idiotype vaccines. I. Generation and characterization of internal image tumor antigen. J Immunol 137: 1743–1749.

Sacks DL, Sher A (1983): Evidence that anti-idiotype induced immunity to experimental African trypanosomiasis

is genetically restricted and requires recognition of combining-site related epitopes. J Immunol 131:1511–1515.

Schick MR, Dreesman GR, Kennedy RC (1987): Induction of anti-hepatitis B surface antigen response in mice by non-internal image (Ab2a) anti-idiotypic antibodies. J Immunol 138:3419–3425.

Sharkey RM, Kaltovich FA, Shih LB, Fand I, Govelitz G, Goldenberg DM (1988): Radioimmunotherapy of human colonic cancer xenografts with Y-labeled monoclonal antibodies to carcinoembryonic antigen. Cancer Res 40:3270–3275.

Shively JE, Beatty JD (1985): CEA related antigens: Molecular biology and clinical significance. CRC Crit Rev Oncol Hematol 2:355–357.

Stein K, Soederstroem T (1984): Neonatal administration of idiotype or anti-idiotype primes for protection against *Escherichia coli* K13 infection in mice. J Exp Med 160: 1001–1011.

Thompson JA, Mauch EM, Chen FS, Hinoda Y, Schrewe H, Berling B, Barnett S, von Kleist S, Shively JE, Zimmermann W (1989): Analysis of the size of the carcinoembryonic antigen (CEA) gene family: Isolation and sequencing of N-terminal domain exons. Biochem Biophys Res Commun 158:996–1004.