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MONOCLONAL ANTIBODIES SPECIFIC FOR CARCINOEMBRYONIC ANTIGEN PRODUCED BY HYBRIDOMA TECHNOLOGY *

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Antibodies against CEA have been used at the Oncologic Center of Nuclear Medicine since 1977 [8, 9]. The tumor and organ specificity of CEA, however, was challenged by several reports describing small amounts of substances immunologically identical to CEA in normal colon mucosa and in carcinoma from non digestive organs. In addition, it has been shown that CEA shares antigenic determinants with crossreacting substances present in large amounts in normal adult tissues [11, 12]. Efforts to improve the specificity of CEA assays by alternative approaches for purification of the antigen and of the antisera have met with only partial success [13, 14]. The adaptation of cell hybridization techniques to the construction of hybrid cell lines producing monoclonal antibodies with desired reactivities has revolutionized the approach to production and utilization of immunospecific reagents [7]. Such a cell line produces a single specie of antibody specific for an individual antigenic determinant regardless of the complexity of the antigen. Thus, individual antigenic determinants associated with CEA preparations might be identified if monoclonal antibodies to CEA are available.

This report describes the production of monoclonal antibodies to CEA by somatic cell fusion of mouse myeloma and mouse spleen cells and demonstrates the potential usefulness of one of these antibodies.

Materials and methods

Production of hybridomas: One month-old female Balb/c mice were immunized monthly by two ip injections of 5 and 10μg of purified CEA in CFA. A series of booster injections were given one month later, with 5μg CEA in PBS being given ev 3 days before fusion, 5μg CEA in CFA given ip 2 days before and 5μg CEA in PBS given 1 day before fusion.

Cell fusion was carried out using 10^8 immunized spleen cells obtained from a mouse showing high anti-CEA titer serum ($>1:10^3$) and 10^7 mouse myeloma P3-X63-Ag 8.653 in 30% PEG (M.Wt. 1500, BDH).

The fused cells were resuspended in RPMI 1640 (Gibco) supplemented with 15% FCS (Gibco), 2mM sodium pyruvate, 20mM Hepes (pH 7.2), 2.2mM sodium bicarbonate, 100IU/ml penicillin, 100μg/ml streptomycin, 10^{-4}M hypoxantine, $4x10^{-7}$M methotrexate, $1.6x10^{-5}$M thymidine (HMT medium) [6] and distributed into each well of two 24-place tissue culture plates (Falcon Plastics) containing feeder layers of $5x10^4$ Balb/c peritoneal macrophages, and grown at 37°C in a 5% CO_2 humidified incubator.

Positive hybridomas were cloned at limiting dilutions in 96-well microtiter plates (Nunc) containing 10^4 feeder layers. After repetitive minicloning and expansion, they were frozen in liquid nitrogen. In order to obtain larger amounts of antibody, 10^7 cloned cells, readapted from HMT to normal culture medium, were injected ip into Balb/c mice which had been primed with 0.3ml tetramethylpentadecane (Pristane, Sigma). Animals were observed daily and paracentesis was performed every 2 to 3 days after appearance of ascitis. Ascitic fluids

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Abbreviations used: CEA, carcinoembryonic antigen; MAB, monoclonal antibody; PBS, 0.15M NaCl and 0.01M phosphate buffer pH 7.4; CFA, complete Freund's adjuvant; SDS-PAGE, sodium dodecyl sulphate-polyacrilamyde gel electrophoresis; BSA, bovine serum albumin; MTX, methotrexate; AMT, aminopterin; FEG, polyethilenglycol; FCS, fetal calf serum; RAMIG, rabbit anti mouse immunoglobulin.

were centrifuged at 500g for 5min, the pellets were reinjected ip or sc, and the supernatants conserved at 4°C with sodium azide.

Monoclonality was confirmed by internally labelling antibodies with ^{14}C-lysine followed by SDS-PAGE and autoradiography.

Antibody detection assay: Culture fluids of growing hybridomas were tested for the presence of anti-CEA antibody by a modification of a commercial CEA RIA (PHADEBAS CEA PRIST). Purified CEA (50µl, 500µg/ml) with a characteristic single broad band on SDS-PAGE (not shown) was incubated overnight with anti-CEA antibodies covalently coupled to paper discs. After washing, supernatants (aprox. 100µl) were taken from wells containing growing hybrids and incubated 6h with the paper discs. The discs were washed again and a second antibody binding reaction was performed adding 50µl of ^{125}I-RAMIG (approx. 50 000cpm, labelled by Iodogen method) [3] diluted in PBS-BSA 1%-azide (RIA buffer) for 18h at 4°C. The washed papers were counted in a Packard Gamma Counter. Positive wells had at least 4 times the nonspecific radioactivity bound by a monoclonal antibody used as control.

Cross-over immunoelectrophoresis: A radioimmunological method carried out on agar gel in veronal buffer, pH 8.2, was applied to determine the specificity of the antibodies [2]. A known amount of ^{125}I-CEA and antibody placed in parallel slots cut in the gel were electrically driven toward each other (300V; 5mA per slide) in a Shandon apparatus. The radioactivity in the reaction zone was determined by γ-counting.

Immunoscintigraphy: Mice bearing an anti-CEA producing solid hybridoma implanted sc, were pretreated for 4 days with Lugol's solution and then given ^{125}I-CEA (50µCi, labelled by Iodogen method) endovenously. Total body images were obtained at 1, 24, 48, 72 and 144h with a Technicare Corp. Gamma Camera, using a high resolution collimator. Images of 20 000 counts were obtained and processed by a MCS 560 computer system [15].

Results

Of the original 48 wells containing fused cells, 31 (65%) contained growing hybrid cells after 7 days. Anti-CEA antibody production was detected in 4 (13%). Line 2B4, which was selected for cloning by limiting dilution in 96-well plates, showed growing cells in 63 (65%), and antibody production was detected in 25 (40%). Eleven of these, stabilized by repetitive minicloning, were saved as frozen specimens. The subcloned lines A4, A15 and A19, all of them of the IgG_1 isotype, were injected intraperitoneally into Balb/c mice where they produced ascitic tumors secreting large amounts of anti-CEA antibodies used for subsequent studies.

To confirm the specificity of the immune reaction for CEA, cross-over electrophoresis were carried out. Figure 1 *a)-d)* shows that

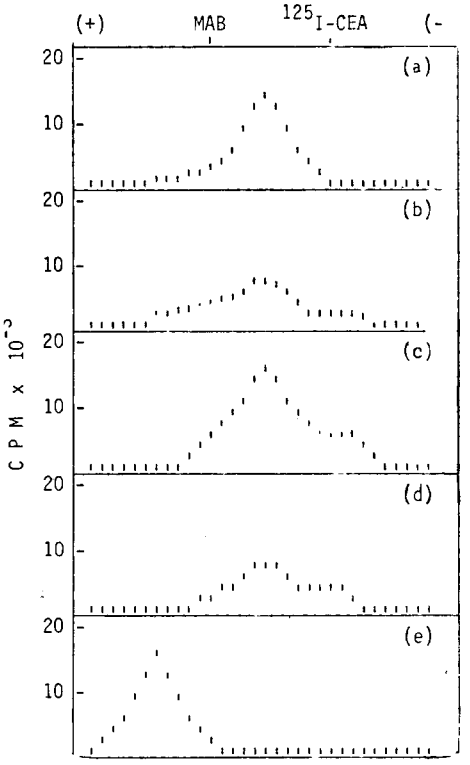

Fig. 1. — Schematic representation of cross-over electrophoresis in agar-gel. The profile curve of radioactivity in agar strips is shown for anti-CEA MAB A4 **a)**, A15 **b)**, A19 **c)** and for mouse anti-CEA serum **d)** and control mouse serum **e)**.

^{125}I-CEA was immunoprecipitated by all three MABs as well as by a mouse anti-CEA serum.

The double-antibody solid-phase assay was used to titrate the binding of MAB to CEA. A representative titration curve for ascitis fluid A4 showed that a high content of antibody (14mg/ml) determined from the absorbance at 280nm ($E_{mg/ml}$=0.71) inhibited ^{125}I-RAMIG binding. At dilutions greater than 1 : 10^4 the binding of the second labelled antibody decreased with anti-CEA MAB concentration. The antibody activities of ascitis fluids were found to be generally 100-1000 times greater than that shown by the antibodies obtained from spent culture medium.

In competition assays of MAB with labelled polyclonal antisera for immunizing type of CEA, inhibitions of 35.1% for A4, 28.1% for A15 and 29.5% for A19 were observed. The degree of suppression of polyclonal antibody binding to CEA obtained from colorectal cancer patients sera, ranged from 10 to 15%.

TABLE 1. — *A comparison between radioimmunoassay for CEA kit and MAB-A4* *

Primary Tumor	n	CEA Kit (normal value<10ng/ml)		MAB-A4 (normal value<200cpm)	
		Positive (%)	Range (ng/ml)	Positive (%)	Range (cpm)
Gastrointestinal	15	12 (80.0)	55-980	12 (80.0)	500-4840
Others	18	8 (44.4)	26-284	0 (0.0)	—

* Serum samples were obtained from patients treated at the Instituto de Oncología A. H. Roffo. Standard samples with known amounts of antigens were treated in the same way as the samples from serum. All samples were assayed in duplicate. For details see Materials and methods.

The affinity constant of antibody from clon A4 was determined by measuring the binding of a limiting amount of antibody to increasing amounts of purified CEA (from 0.5 to 500ng/ml (Fig. 2). The transformation of the saturation curve into single reciprocal plot (Fig. 2, insert) were used to determine the affinity constant (K_a). The plot, with a calculated affinity of 1.60×10^{10} liters/mol, was linear, as expected for homogeneous MABs.

MAB A4 was applied in preliminary experiments to detect the antigen in serum from diseased and healthy individuals. When patients with elevated CEA levels were analized, only 12 out of 20 showed positive results with MAB, all of them detected in sera from patients with gastrointestinal carcinoma. The remaining eight tumors were of breast, lung and liver origin. Normal values obtained with the commercial kit (n=13) were reproduced by the "monoclonal assay".

The potential of antitumor MABs for diagnostic purpose include not only the detection of the tumor associated antigens in serum. Another approach involved localisation of tumor deposits by Gamma Camera scintigraphy following injection of a radioisotope probe [10]. Accordingly, in order to determine if MAB A4 reacts in vivo with CEA, mice bearing a subcutaneous deposit of A4 hybridoma were injected endovenously with ^{125}I-CEA and imaged during six days (Fig. 3,

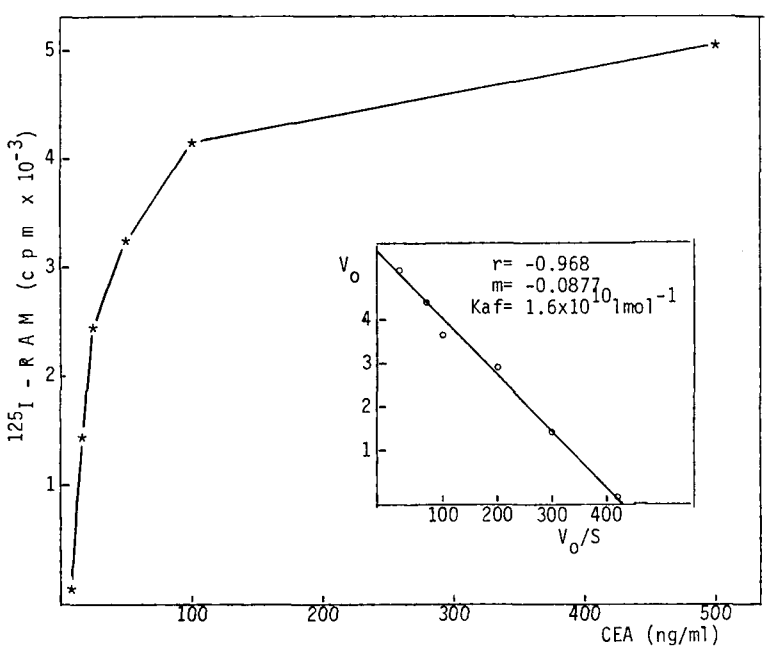

Fig. 2. — Standard curve for the detection of CEA by the solid-phase radioimmunoassay. Figure within the graph represents the best fit of the experimental data to the linear relation $V/S = \frac{1}{Km} + \frac{Vm}{Km}$ (Eadie-Hofstee plot).

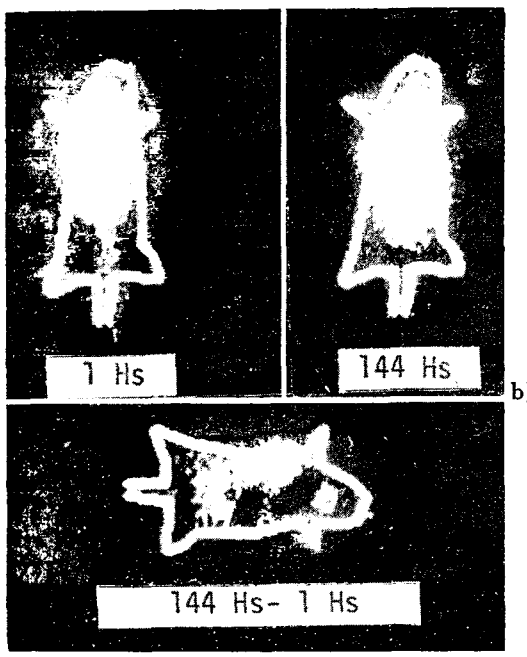

Fig. 3. — Gamma camera scintigraphy of tumor localisation with ^{125}I-CEA in mice bearing subcutaneous A4 growth.

a)-c)). The visualisation of the tumor mass can be seen in Fig. 3c), after computarized substraction of ^{125}I-CEA blood levels.

Discussion

A stable line of mouse myeloma-spleen cell hybrid producing antibodies against CEA has been isolated. The frequency of antigen specific hybridomas (13%) was rather high compared with those reported by other groups raising MAB to soluble antigens, such as CEA [1, 4]. Though the parameters that determine the optimal immune response and yield of hybrids remain poorly understood, the obtained frequency appears to be related to the mouse immunisation regime [16] as well as the use of MTX, in place of AMT, for the rescue of hybrid cells in selective medium due to its advantages to preserve stable clones [6].

All eleven cloned hybrid cell lines, stabilized by repetitive minicloning, showed greater than 95% positive wells, indicating that a single uncontaminated anti-CEA secreting clon was isolated during the first cloning procedure.

The specificity of the MAB determined by the solid-phase RIA was further evidenced by cross-over immunoelectrophoresis experiments. The immunoprecipitation of labelled CEA by MAB A4, A15 and A19 and the lack of cross reaction with control MAB, as shown in Figure 1c), indicated that the antigenic determinant(s) recognized by the MAB were associated with CEA, although the precise epitope has not yet been defined.

Although the solid-phase RIA seems to be the most obvious immunoassay to measure anti-CEA activity, other modifications may be suitable as well as confirmation test for positive samples or to compare the binding of MAB with that achieved using the commercial polyclonal antibody. Data from competition-assays showed that pretreatment of purified or serum CEA with MAB produced an specific and consistent binding suppression of the antigen to the polyclonal antibody on the paper disc. Therefore, the hybrid cells might be producing an antibody recognizing a reduced spectrum of antigenic determinants in comparison with those identified by the conventional polyclonal antibodies.

The calculated affinity constant (K_a=1.60x 10^{10} liters/mol) for MAB A4, which compared favourably with those reported by other workers [1, 4], suggests that hybrid clone A4 is likely to secrete antibodies of high affinity. Clones producing less avid antibodies avoided detection with our 6h-solid-phase RIA due to the shorter half-lives of antigen-MAB complexes [16].

As a prelude to human experimentation, patients serum samples were analyzed by RIA. Among 20 patients with high levels of CEA, only 12 were positive with MAB A4. Since MAB A4 selectively reacted against CEA present in gastrointestinal cancer patients, our MAB seems to recognize CEA epitope(s) apparently not shared by closely related molecules already present in tumors from other sources.

Finally, the in vivo localisation and imaging of the labelled probe ^{125}I-CEA in subcutaneous A4 hybridoma tumor gave us additional evidence that MAB A4 reacts with isolated CEA as well as with the antigenic molecule *in situ*. Although more patients must be studied before the precise clinical utility of MAB A4 is completely defined, given the results obtained, it may have real value as a diagnostic probe.

Summary

This report describes the production of monoclonal antibodies to CEA by somatic cell fusion of mouse myeloma and mouse

spleen cells and demonstrates the potential usefulness of one of these antibodies. Mice were immunized monthly by two ip injections of CEA and boosted with three consecutive injections on days 3, 2 and 1 before fusion. Cell fusion was carried out using immunized spleen cells and mouse myeloma P3-X63-Ag8.653 in 30% PEG. Fused cells were resuspended in HMT medium and distributed into 24-wells culture plates containing feeder layers of Balb/c peritoneal macrophages. Culture fluids of growing hybridomas were tested for the presence of anti-CEA antibodies by RIA. Of the original 48 wells containing fused cells, 31 (65%) contained growing hybrid cells and 4 (13%) showed anti-CEA antibody production. Line 2B4 was selected for cloning by limiting dilution and the subcloned lines A4, A15 and A19 were injected ip to obtain ascitic tumors. The specificity of the anti-CEA antibodies was confirmed by cross over electrophoresis using ^{125}I-CEA. Titration curve of ascitic fluid A4 showed that high content of antibody inhibited the binding of ^{125}I-RAMIG, while at dilutions greater than 1 : 10^4 the binding decreased. In competition assay of MAB with labelled polyclonal antisera for purified CEA, inhibitions ranging from 28 to 35% were observed. Colorectal cancer patients' sera produced suppression from 10 to 15%. The affinity constant of MAB A4 was determined by measuring the binding of a limiting amount of MAB to increasing amounts of CEA. The transformation of the saturation curve into reciprocal plot, with a calculated affinity of 1.69×10^{10} liters/mol, was linear, as expected for homogeneous antibodies.

In order to determine if MAB A4 reacts in vivo with CEA, mice bearing a sc deposit of A4 hybridoma were injected ev with ^{125}I-CEA and imaged by gamma camera scintigraphy during six days. After computarized substraction of background ^{125}I counts, the visualisation of the tumor mass was achieved.

Preliminary experiments were carried out to detect CEA in sera from diseased and healthy individuals. When patients with elevated CEA levels were analized, only 12 out of 20 showed positive results with MAB, all of them in sera from patients with gastrointestinal cancer. Normal values obtained with the commercial kit (n=13) were reproduced by the "monoclonay assay".

Resumen

ANTICUERPOS MONOCLONALES ESPECÍFICOS PARA EL ANTÍGENO CARCINOEMBRIONARIO PRODUCIDOS POR LA TÉCNICA DE HIBRIDOMAS.

El objetivo del presente trabajo fue la producción, caracterización y aplicación de anticuerpos monoclonales anti-antígeno carcinoembrionario. Ratones Balb/c fueron inmunizados con 2 dosis ip de 5 y 10μg de antígeno carcinoembrionario en adyuvante completo de Freund cada 30 días, y 3 dosis de refuerzo durante días consecutivos. Las células esplénicas fueron fusionadas con células de mieloma X63/Ag8.653 en presencia de polietilenglicol, y los híbridos rescatados en medio selectivo conteniendo hipoxantina-methotrexate-timidina. La detección de híbridos productores de anticuerpos monoclonales dirigidos contra el antígeno se realizó por radioinmunoanálisis en fase sólida, utilizando como trazador inmunoglobulina de conejo anti-ratón marcada con ^{125}I. Se obtuvo una eficiencia del 65% en crecimiento y del 13% en positividad. Los híbridos fueron clonados y estabilizados por miniclonado repetitivo, con un 65% de crecimiento y 40% de positividad. De 11 hibridomas productores se seleccionaron 3 que fueron inyectados en ratones Balb/c para la obtención de tumores ascíticos. La inmunoespecificidad de los anticuerpos monoclonales A4, A15 y A19 fue determinada por inmunoelectroforesis crczada, detectándose la formación de inmunoprecipitados específicos entre los anticuerpos monoclonales y el antígeno carcinoembrionario marcado con ^{125}I. El anticuerpo monoclonal A4, de alta afinidad ($K=1{,}60 \times 10^{10}$ litros/mol) fue activo en el radioinmunoensayo hasta la dilución 1 : 10 000 testada, permitiendo detectar valores de aproximadamente 0,5ng/ml. En ensayos de competición contra el anticuerpo policlonal, el rango de inhibición obtenido fue del 28 al 35% utilizando antígeno purificado, y del 10 al 15% con sueros de pacientes afectados por cáncer de colon.

En ratones portadores de hibridoma productor del anticuerpo monoclonal A4 implantado en forma subcutánea, la inyección de antígeno carcinoembrionario marcado con ^{125}I permitió la localización gammagráfica del tumor.

Resultados preliminares de dosaje de antígeno carcinoembrionario en pacientes portadores de cáncer, arrojaron valores normales (n=13) en coincidencia con el kit comercial,

mientras que sólo 12 de 20 pacientes con valores elevados en el ensayo habitual son positivos para el anticuerpo monoclonal, todos ellos portadoses de tumores gastrointestinales.

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