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⑲ ⒸⒶ # CANADIAN PATENT

㊾ METHOD FOR DETECTING THE PRESENCE OF
CARCINOEMBRYONIC ANTIGEN IN BLOOD

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The neoplastic process in human beings has been and still is the subject of intensive study. In order to obtain a better understanding of the disease, human cancer tissue has been studied in an effort to discover the cause, treatment, prevention and/or diagnosis of cancer. Early diagnosis of cancer is very important since it increases the chances of effecting a complete remission of the disease.

In an effort to utilize known diagnostic tools to detect the presence of cancer tumors, attempts have been made to demonstrate tumor specific antigens to human carcinoma. These attempts have previously been unsuccessful since it has not been possible to segregate normal tissue antigens from abnormal cancer antigens and demonstrate that the cancer antigens are specific.

In the attempts to isolate abnormal cancer antigens and demonstrate their specificity, attempts have been made to cause the formation of tumor-specific antibodies and demonstrate their presence in sera obtained from animals immunized with preparations of human cancer. If consistently reproducible, the demonstration of the presence of tumor-specific antibodies in animal antisera would lead to the use of a valuabe diagnostic tool.

In order to fully utilize the existence of tumor-specific antibodies in animal antisera as a diagnostic tool, a test must be developed which will demonstrate the presence of the tumor-antigen in the blood of the patient. Previously devised procedures have not proven efficient or effective in the detection of digestive system cancer.

Among the human carcinoma most extensively studied by investigators is adenocarcinoma of the colon and digestive tract, since this is one of the most wide-spread cancers and usually requires a surgical procedure for definitive diagnosis after some gross symptomatology has developed.

Previous efforts to extract an antigen associated with colonic cancer have been unsuccessful since it has not been possible to completely segregate it from normal tissue antigens. Indeed, it had not been demonstrated that such an antigen did in fact exist.

We have, however, demonstrated the presence of antigens which are

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specific to adenocarcinomata of the colon and digestive system by means of immunological tolerance and absorption techniques, Gold et al., J. Exptl. Med. 121 439-462 (1965). However, the practical isolation of the antigen itself had until the present invention not been achieved.

These tumor specific antigens have been found by us to be present only in patients who have adenocarcinoma which originate in digestive system epithelium derived from embryonic entodermal tissue, i.e., esophagus, stomach, duodenum, pancreas and rectum.

We have also previously demonstrated that the tumor specific antigen is also present in the digestive organs of fetuses between two and six months of gestation, Gold et al., J. Exptl. Med. 122 467-487 (1965). Thus, for convenience, we have designated the antigen as carcinoembryonic antigen (CEA) of the human digestive system.

Not only has it been heretofore not possible to isolate and characterize the CEA but it has not been possible to demonstrate its presence in the blood of persons having adenocarcinoma of the colon. This is true because the antibodies formed in response to the CEA combine with the CEA into a complex, thus removing the antigen from the blood. Methods, therefore, had to be devised to destroy the antibody-antigen complex without altering the antigen determinant sites. Further, once this had been accomplished, it became necessary to find a means of tagging CEA so it can be detected.

DETAILED DESCRIPTION OF THE INVENTION

According to this invention, we have discovered

(a) CEA and methods that enable its isolation, purification and confirmation of its identity and specificity;

(b) methods of producing specific antibodies for CEA;

(c) methods for tagging CEA and the novel tagged CEA;

(d) methods for utilizing the tagged CEA to detect the presence of adenocarcinoma originating in digestive system epithelium derived from embryonic entodermal tissue; and

(e) methods for treating serum to dissociate CEA-anti-CEA complexes.

The carcinoembryonic antigen is isolated and purified by homogeniz-

ing adenocarcinoma tissue from primary or metastatic tumors originating within the digestive system.

In order to isolate the antigen associated with the homogenized tumor tissue, it is necessary to separate all other material from the homogenate. This is accomplished by chemical and physical extraction and purification procedures.

Once the extraction and purification procedures are completed, the identity of the isolated fraction as CEA must be confirmed. This is accomplished by various techniques known in the art, e.g., double diffusion in agar gel, innunoelectrophoresis, hemagglutination, passive cutaneous anaphylaxis and the like.

In order to utilize these techniques, the antibodies used must be confirmed to be specific for the CEA. Antibodies which meet this criteria can be produced by immunological tolerance or absorption techniques.

In the absorption technique, antitumor antiserum is absorbed with normal tissue in order to remove anti-normal components of the antiserum. Any residual antibody activity in the absorbed antiserum which is directed against tumor material is then considered to be tumor specific. This method is not without its faults since there is the possibility that tumor specific antibodies may have been removed or inactivated by normal tissue components similar to, but not identical with, the tumor antigens which initially stimulated the antibody production.

In the immunological tolerance technique, animals are rendered immunologically tolerant to normal tissues during neonatal life. The tolerant animals are then immunized with tumor preparations of the same donor species. Where adequate suppression of the immune response to normal tissue components has been achieved, the development of antibodies apparently specific for the tumor has been achieved. In studies of human cancer, this technique is open to possible misinterpretation since the source of the normal and tumor material are different donors. However, if extracts of the tissues are used, this problem is not significant since the extracts are all substantially similar.

We have found that colon adenocarcinoma tumor tissue and normal colon tissue from the same individual can be utilized because carcinoma of the colon almost never extends submucosally more than 6 to 7 cm. on either side of a tumor visible in the gross.

The colon adenocarcinoma tumor tissue and normal colon tissue from the same individual are treated separately but in parallel fashion. The tissue is ground up, suspended in a buffer, then homogenized. The homogenate is then treated to remove solid particles. Centrifugation or filtering through successively smaller filter openings are preferred. The purpose is to remove all particles about 0.22 μ or larger, thus removing all the bacteria present. The supernatant or filtrate is thereby sterilized to insure against bacterial contamination.

Test animals divided into appropriate groups are then immunized with the extracts and after a suitable time interval serum is obtained from the animals. The presence of antibodies in the test sera is demonstrated by either the Ouchterlony technique of double diffusion in agar gel, immunoelectrophoresis, hemagglutination reactions or passive cutaneous anaphylaxis. The preferred practical method, because of its simplicity and reproducible results is the Ouchterlony technique.

Once the antibodies are demonstrated to be present, it is possible to determine if a particular extraction technique does, in fact, isolate a fraction which contains carcinoembryonic antigen.

We have discovered an extraction and purification technique which results in a fraction which invariably produces one precipitant line in the Ouchterlony technique when tested against the antibody which is specific for CEA or when tested against non-absorbed antisera.

As stated previously, the CEA is isolated and purified, according to this invention, from primary or metastatic adenocarcinoma tissue which originates in the digestive system epithelium that is derived from embryonic entodermal cells. Also, the CEA can be isolated and purified according to this invention from embryonic digestive organs of fetuses in the second to seventh month of gestation. The following description will be directed to

extraction from cancer tissue, however, the process may apply also to embryonic tissue.

Tissue containing CEA, either embryonic digestive organ tissue from the first and second trimesters or adenocarcinoma tissue of tumors which originated within the digestive system epithelium as described above are extracted with a glycoprotein solvent in which CEA is soluble. This is required so that precipitable normal proteins and interfering antigenic materials can be separated from the CEA material. Glycoprotein solvents which are suitable are, e.g., perchloric acid, trichloroacetic acid, phosphotungstic acid and the like. However, perchloric acid, because of its availability and ease of use is preferred. The tissue which is being treated is homogenized with water in order to solubilize the antigen. The amount of water used should be sufficient to solubilize all of the carcinoembryonic antigen. Generally about two liters of water per about every kilogram of tissue is sufficient. More water can be used, however, it is usually not necessary.

Any temperature below room temperature is suitable for the addition of the glycoprotein solvent to the tissue homogenate. Preferably, however, room temperature, e.g., 20°C. to 25°C. is utilized. The temperature of the glycoprotein solvent which is added to the tissue homogenate can also be variable, however, room temperature is preferred. Generally, a concentrated acid is used as the glycoprotein solvent, e.g., about 0.5 N to about 2 N, with 2 N being preferred. The solvent is added in about equal volume to the homogenate. The time in which this is accomplished is usually from about 10 to about 30 minutes. Longer times can result in loss of antigenicity.

A precipitate results. This precipitate is separated from the supernatant containing the dissolved CEA. Any convenient method of separation is suitable, e.g., filtration, centrifugation and the like.

Centrifugation is preferred because it is faster and sufficient force can be developed to remove substantially all the solid particles. Generally, about 3,000 to about 8,000 revolutions per minute are sufficient to accomplish this. This centrifugation is preferably carried out at cold temperatures, e.g., about 4°C. to about 10°C. since at these temperatures the time for sedi-

mentation is substantially decreased.

Perchloric acid, salts such as sodium chloride and other low molecular weight materials are then removed in order to further purify the system. While it may be possible to accomplish this by precipitating the remaining proteins, it has been discovered that dialysis through a semipermeable membrane against water is most suitable. The dialysis is a critical part of the process since it eliminates all diffusible soluble materials except the higher molecular weight materials which include the CEA fraction. The dialysis can be carried out initially with tap water for about twenty-four to forty-eight hours. Then subsequent dialysis can be carried out with cold distilled water at temperatures of about 4°C. to about 10°C. However, it is possible to use only distilled water in the dialysis provided the temperature remains low and the water is changed frequently. The complete dialysis procedure takes about 48 to 96 hours.

The cloudy solution which remains after the dialysis is then dried. Spray drying can be used, however, lyophylization is the preferred method to remove water. Any conventional lyophylization process is suitable. The preferred process is wherein the material is first shell-frozen then lyophylized. It usually takes about 32 to 36 hours for the product to be lyophylized to complete dryness. However, in a preferred embodiment of the process of this invention, the lyophylization is carried out for about 24 to 30 hours so the product is not completely dry. While this step in the process is not critical to the eventual isolation of pure CEA, it is preferred since it saves time.

The partially lyophylized material can be thawed out at room temperature or at higher temperatures, e.g., about 37°C. It can also be thawed by diluting with a minimum amount of water and then the remaining particles are removed. It has been found that when the volume is kept to a minimum, the clarification can be accomplished by filtration and/or centrifugation. All particles of a size which will not pass through 0.22 μ filters (the size of the smallest bacteria) are removed thus clarifying and purifying the solution.

The preferred process is wherein the solution is centrifuged at

high speed, i.e., from about 14,000 to 30,000 rpm, preferably about 14,000

rpm at temperatures below about 10°C., i.e., about 4°C. to about 10°C., for

about 15 to 45 minutes. Higher temperatures are operable, however, they de-

crease to sedimentation rate and are thus not practical. After centrifugation,

it is necessary to further clarify and sterilize the supernatant. This can

be accomplished by filtering it sequentially through filters of diminishing

pore size to remove substantially any solids and bacteria remaining. The pore

sizes of the filters can vary from about 1.2 µ to about 0.20 µ. Preferably,

filters with 1.2 µ, 0.45 µ and 0.22 µ are used in the order listed.

10   The separation of the portion of the resulting lyophylized powder

containing CEA to the exclusion of other materials is accomplished according

to this invention by chromatography with two different gel columns followed

by electrophoresis.

We have found that the eluted fractions from the column chromato-

graphy which have a molecular weight of about 200,000 and a definite peak at

the spectrophotometric absorption wave length of 280 µ are those containing

the CEA. We have further discovered that when the CEA containing fractions

are subjected to electrophoresis, they have a unique migration pattern which

is anodal to the application zone at the cathode at pH 8.6 with a borate buf-

20   fer having an ionic strength of 0.05. The amount of migration depends upon

the current and voltage used as well as the pH, buffer and ionic strength of

the buffer. For example, when 400 volts and 20 mA are used, the CEA migrates

anodally about 10-14 cm. to the application zone at the same time that ferri-

tin, a colored protein, used as a marker when applied to the cathodal extrem-

ity migrates 18 cm. anodally. Other markers can be used, however, the elec-

trophoresis conditions will then have to be changed and the migration of the

CEA and marker correlated.

The column chromatography can be accomplished by subjecting the lyo-

phylized material, in solution, to sequential chromatography on two different

30   gel columns in any order. Practically, however, a gel column which is used

in accordance with this invention is an agarose gel. Agarose is the neutral

portion of agar. The gel material which is commercially available from AB

Pharmacia Uppsala, Sweden under the trade mark "Sepharose". The gels are available as aqueous suspensions in 0.02% sodium azide as a preservative. The gel structure is due to hydrogen bonding. The gel is prepared in beaded form having a selected particle size and percent agarose. The concentration of the agarose in the gel determines its fractionation range.

The gels most suitable for use in this invention are those which have a particle size of from 40-190 microns and contain 4% by weight agarose. These materials named "Sepharose 4B" have a fractionation range of 3×10^5 to 3×10^8. In the process of this invention, Sepharose 4B is used since it permits the separation of the CEA containing fraction from extraneous materials of higher and lower molecular weight as well as colloidal particles.

The second column contains a gel filter material which is a hydrophilic water-insoluble cross-linked dextran polymer gel. This material and the method of its manufacture are described in British Patent No. 854,715. The gel material, which is commercially available from AB Pharmacia, Uppsala, Sweden, under the name "Sephadex"; comprises a three dimensional macroscopic network of dextran substances bonded or cross-linked together, being capable of absorbing water with swelling. The ability of the gel material to take up water is inversely proportional to the degree of cross-linkage of dextran substances in the gel material. The gel material is available in a variety of grades differing with respect to degree of porosity. The gel preferred for use in this invention has an approximate molecular weight exclusion limit of 200,000, a water regain (g H_2O/g dry gel) of 20 $\pm$ 2.0 a particle size of 40-120 microns and a bed volume/ml./g. dry gel of 30-40, the gel is named "Sephadex*G-200".

Sephadex*G-200 is employed to further purify the CEA containing fraction. Since the column has greater resolving power than the first column for the molecular weight range of 150,000 to 250,000, further separation of the CEA from higher and lower molecular weight materials is achieved. The second column, for all practical purposes, should be used only after the colloidal particles are removed by the first column since these particles will

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clog the column and make it ineffective. The chromatography is accomplished by dissolving the lyophylized material in an aqueous buffer at the pH wherein the carcinoembryonic antigen is electrically neutral, i.e., pH 4.5, then running the solution through the first column, collecting, dialyzing as described above and lyophylizing the active fraction to remove salts, then redissolving the collected active fraction in an aqueous buffer at the pH wherein the carcinoembryonic antigen is electrically neutral and running the solution through the second column, collecting the active fractions, dialyzing as before and lyophylizing. The reason the carcinoembryonic antigen is made electrically is

10   to minimize the interaction of the antigen with the columns and subsequent superimposition of extraneous in the active fractions. While any convenient aqueous buffer is suitable, it is preferred to use a phosphate buffered saline (0.9%) solution. The advantage of utilizing low temperatures, i.e., from about 4°C. to about 10°C. is that it results in increased resolution. The fractions collected from the second column are those which have a molecular weight of 200,000 and a reading with a peak at 280 mμ on an UV absorptionmeter. These fractions collected from the first column are selected based on the same criteria, however, thy contain materials slightly greater and slightly less (as low as 70,000 MW) than 200,000 MW.

20   The active fractions from the second column are subjected to electrophoresis in order to further fractionate the CEA from impurities. This is accomplished by dissolving the active fraction, previously concentrated, in a buffer of a pH of about 8.2 to 9.2 and an ionic strength of about 0.0125 to 0.10. While any aqueous buffer with these characteristics is suitable, it is preferred to use a buffer which will increase mobility of the active fraction, i.e., a borate buffer of about pH 8.6 and ionic strength of about 0.05. It is these properties which make the migration of the CEA the same as the preferred marker used, i.e., ferritin.

The current and voltage which is used determines the extent of separation of the molecules in the fraction. It has been found that in block

30   electrophoresis, the preferred method, about 300 to 500 volts, preferably 400

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volts, is suitable with a current of about 20 mA. Other currents and voltage can be used, however, those discussed above are preferred.

Block electrophoresis is the preferred electrophoresis method because it facilitates the recovery of the desired fraction. However, other types of electrophoresis can also be used.

The material used in the block electrophoresis should support the material being tested and conduct electricity when impregnated with a buffer. Typical suitable materials are paper and gels. In the preferred electrophoresis method a cross-linked dextran gel, e.g., "Sephadex*G-25" fine having an approximate molecular weight exclusion limit of 5,000, a water regain (g H_2O/ g dry gel) of 2.5 $\pm$ 0.2, particle size of 20-80 microns and a bed volume/ml/g dry gel of 5, is swollen in the buffer and placed on a non-conductive block, e.g., Lucite*

In the preferred method, the gel with the same buffer as the active fraction is dissolved in is placed in the block then paper contacts are placed on the gel. The block is then equilibrated in the same buffer in an electrophoresis apparatus under operating conditions. After equilibration is accomplished, a strip of the gel is removed and mixed with the buffered lyophylized CEA fraction from the second column. The resulting slurry is then poured onto the block at the same place from where the strip was removed, preferably at about the center of the block. The suitable marker, e.g., ferritin, a protein containing iron, is then spotted at the cathodal extremity of the block. After operating the electrophoresis for a predetermined length of time, in this case about 24 hours, the area containing CEA activity, i.e., 10-14 cm. anodal to the application zone, is removed. This area is removed only if the marker has moved a previously determined distance. In this case, 18 cm. anodally. The CEA activity is eluted under suction through a disposable 0.20 μ cellulose acetate filter and recovered, preferably by dialysis against distilled water to remove the salt used in the elution. This results in a material that is substantially pure CEA. This is shown by either the precipitin inhibition or direct Ouchterlony testing against unabsorbed tumor

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antiserum. A single line precipitate indicates pure CEA activity.

In another aspect of this invention, we have discovered a method of determining the levels of carcinoembryonic antigen in body fluids of persons either with cancer or suspected cancer. More particularly, we have discovered a radioimmunoassay technique which is simple to perform and has a high degree of reproducibility and specificity.

In radioimmunoassays, it is important that the radioactive atom be sufficiently reactive with the molecule to be tagged to provide an adequate concentration of radioactivity for determination and the radioactive atom must provide a sufficient number of disintegrations per unit of time to provide sufficient sensitivity for accurate determinations. Further, in the case of radioimmunoassay of antigens, the antigenicity must not be deleteriously affected by the conjugation of the radioactive atom to the antigen.

We have discovered, according to this invention, that for the first time the existence of human tumor growth can be determined by assaying a circulating tumor-specific antigen. By means of the present invention, it is possible to detect at least about 1.0 ng. of CEA per ml. of serum. The sensitivity of the assay is limited only by the specific activity of the radioactive atom. This limitation, however, is largely overcome since virtually all of the CEA present in a particular volume of serum can be extracted by our extraction procedures, thus, making it possible to concentrate the specific activity of the radioactive atom. Further, the coprecipitation-inhibition of the tagged CEA can be augmented to increase the sensitivity of the assay.

The CEA can be tagged with radioactive atoms which will react with its chemically reactive groups and not substantially diminish its antigenicity. We have found ^{125}I to be particularly suitable.

The CEA can be radioiodinated by methods known in the art, with minor modifications to concentration and volumes. The Chloramine T method of Hunter and Greenwood, Biochem J. 91, 46 (1964) using iodine 125 is particularly useful.

This process results in a radioiodination efficiency of about 20 to

50%. In radioiodinating CEA, a purified CEA is used. The reaction is effect-
ed, for example, by using a 200 µl. reaction mixture containing 100 µg. of
Chloramine T (sodium p-toluenesulfo-chloramine); 0.25-0.4 mg. of purified CEA,
and 4 mCi of I^{125} in the form of KI or NaI. The reaction takes place in about
1 minute at room temperature and is stopped by the addition of sodium metabi-
sulfite. The function of the Chloramine T is to oxidize the iodide salt to
iodine. The function of the sodium metabisulfite is to reduce unreacted ^{125}I
back to its salt, other reducing agents can also be used, e.g., potassium meta-
bisulfite. The oxidizing and reducing agents used should not be so strong
10   that they damage the CEA. The product can be separated from residual unreact-
ed I^{125} by chromatography in a cross-linked dextran gel column, e.g., "Sepha-
dex G-100", a gel having an approximate molecular weight exclusion limit of
100,000, a water regain (g H_2O/g dry gel) of 10.0 $\pm$ 1.0, a particle size of
40-120 microns and a bed volume/ml./g. dry gel of 15-20, and removing the tube
with the greatest radioactivity in the first peak. The product has the follow-
ing characteristics:

It is reactive with a specific antibody, has the CEA electrophoresis
pattern, has a molecular weight of about 200,000 and has a specific activity
of between about 1,000-25,000 dpm/ng., preferably between about 10,000 and
20   20,000 dpm/ng., i.e., about 5-10 mµ Ci/ng. of CEA.

In practicing this invention using radioiodinated CEA, the presence
of CEA in human serum is detected using radioimmunoassay procedures based on
a modification of the technique of coprecipitation-inhibition described by
Farr, J. Infect. Dis. 103, 239 (1958).

It is necessary, in order to achieve success in the aforesaid tech-
nique, to treat the patient's blood in a manner which will insure that all
the CEA, to the exclusion of interfering materials, is in the finally used
serum. This can be accomplished by treating blood serum from the patients
with a glycoprotein solvent which solubilized CEA, and clarifying the result-
30   ing solution.

The glycoprotein solvent which has been found suitable for this pro-
cess is perchloric acid. We have found perchloric acid of 0.2M is preferred

since it removes interfering substances. The resulting solution containing

dissolved CEA, if any is present, is then clarified. The preferred method is

to centrifuge, collect the supernatant and dialyze against water. This usual-

ly takes about 24 to 48 hours. The dialysis residue can then be dried by lyo-

phylization. By using this method a purified serum extract containing greater

than about 95 percent of the CEA originally present is produced.

It is important to this process that the serum extract is treated as

described since the glycoprotein solvent which solubilized CEA in the initial

step dissociates any preexisting CEA-anti-CEA complexes in the patients serum,

10   enabling the recovery of substantially all the CEA originally present.

Further in order to effectively conduct the radioimmunoassay, a sup-

ply of CEA specific antibodies must be assured. This is accomplished by im-

munizing animals with purified CEA in conventional manner.

An emulsifier, e.g., Freunds adjuvant (complete) is added to CEA in

a saline solution. The emulsion can be injected in animals intramuscularly,

subcutaneously, in the foot pad or any combination of these methods. Animals

such as fowl, rabbits, horses, goats, sheep and the like are suitable. The

regimen in rabbits, for example, is injections twice a week until five injec-

tions are made. After the last injection, blood is collected from the animal.

20   The serum from this blood is unabsorbed anti-CEA antiserum.

In one method, 400 µg. of CEA in 1 ml. of saline solution (0.9%) is

utilized. The injection is made intramuscularly using a volume about four

times that injected in the foot pad.

The antibody present in the antiserum, after absorption with normal

tissue components, is specific in its activity against CEA to the exclusion of

other antigens.

In conducting the radioimmunoassay of CEA, procedures based on the

technique of coprecipitation-inhibition are used.

In this process, a titration curve, then a standard inhibition curve

30   are obtained. The standard inhibition curve is used as a control standard,

then the radioactive materials are coprecipitated from the solution.

The standard inhibition curve, which is made by the Farr procedure,

is a measure of the complex formation with specific antibodies. The curve

reflects the amount of CEA present per unit of serum. The measurement is in

nanograms per ml., this is plotted against a known percentage of radioactive

tagged CEA. The resulting curve is used to plot the amount of CEA in a pa-

tient's serum.

A standard inhibition curve is obtained by adding standard CEA to a

series of tubes containing powdered perchloric acid extract of normal human

serum. A measured amount of anti-CEA-antiserum which had previously been di-

luted 1:100 with a borate buffer of about pH 8.4, is added to the series of

10   tubes containing a perchloric acid extract of normal blood serum. The buffer

used to dilute the normal blood serum can be any buffer, however, a borate

buffer with an ionic strength of 0.1, and a pH 8.4 is preferred.

The resulting solutions are incubated at low temperatures, e.g., a-

bout 4°C. for a sufficient time to complete the reaction, usually about 18

hours is sufficient. Following the incubation, a measured amount of I^{125} is

added to each of the tubes. The incubation is then continued for an addition-

al 2 hours at about 37°C. When the incubation is completed, a precipitant

which precipitates the antibody and antigen-antibody complex but not the anti-   .

gen, is added to the solution to coprecipitate the antibody bound CEA. Pre-

20   ferably, a saturated ammonium sulfate solution is used.

Under the conditions described above, free CEA remains in solution.

The I^{125} content of the precipitate or supernatant is then determined from a

reading on a suitable instrument and the amount of CEA in the serum is then

determined by reference to a standard.

The assay performed on the powdered perchloric acid extracts of

serum, processed in the same manner as the standard CEA, results in a deter-

mination of the amount of CEA in the patient's blood. This in turn indicates

the presence or absence of digestive system cancer in the patient.

The following Examples illustrate the invention.

30   Example 1

Isolation of Carcinoembryonic Antigen Fraction

Tumor tissue from adenocarcinoma of the colon is dissected and as

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much normal tissue as possible is discarded. The remaining tumor tissue is
passed through a meat grinder and stored in 1000 gram lots, at -20°C. 1000
Grams of the minced tissue is suspended in distilled water to total volume of
4000 ml., and homogenized at 15,000 rpm in a water cooled homogenizer for 1
hour.

An equal volume of 2N perchloric acid at 4°C. is added slowly, with
constant stirring. This suspension is stirred for 30 minutes at room temper-
ature, and then centrifuged at 6000 rpm at 4°C. for 30 minutes in a refriger-
ated centrifuge (6 x 250 ml. fixed angle rotor). The supernatant is removed

10   and placed in flat 3" wide dialyzer tubing, previously soaked for 1 hour in
distilled water to render it more pliable. Dialysis is carried out against
cold running tap water for 48 hours, and then against continuous changes of
large volumes of distilled water at 4°C. for an additional 48 hours. The di-
alyzed residue is placed in 5000 ml. round-bottom flasks, 1500 ml. per flask,
shell-frozen in a methanol-dry ice bath, and lyophylized on an 8 liter capa-
city lyophylizer. Just prior to complete lyophylization (24-30 hours) the
flasks are removed, the partially dried crude extract is thawed out and the
resulting solution spun at 14,000 rpm at 4° for 30 minutes in a refrigerated
centrifuge (8 x 30 ml. fixed angle rotor). The supernatant is removed and

20   then passed sequentially through 1.2 μ, 0.45 μ and 0.22 μ Millipore* membrane
filters. The clarified solution is shell-frozen and lyophylized to dryness.

1.5 Gms. of dried crude extract are dissolved in 50 ml. of 0.05M
NaH_2PO_4 buffer (pH 4.5) in normal saline. The phosphate saline buffer, pH
4.5, serves as the eluting solution in all subsequent column chromatography.
The sample is then put through a Sepharose 4-B column. The column used is a
Pharmacia K 100/100 (100 cm. x 10 cm.) with a bed length of 89 cm. The temp-
erature is maintained at 4°-5° by a recirculating cooler. The solution is
pumped at an upward flow rate of 150 ml./hr. by a constant flow pump (LKB
"Varioperpex 1200" Peristaltic Pump). 25 Ml. fractions are collected in a

30   fraction collector (LKB "Ultrorac 7000"). The fractions are constantly moni-
tored at 280 μ on UV absorptionmeter. The active fraction is collected after

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4750 ml. are eluted. The run takes approximately 47 hours.

The total volume of the active fraction is 750 ml. The active fractions are pooled, dialyzed for 48 hours at 4° against several changes of large volumes of distilled water shell-frozen and lyophylized.

200 Mg. of dried material from the Sepharose 4-B column is then dissolved in 10 ml. of standard buffer and put through a Sephadex G-200 column. The column used is a Pharmacia K 50/100 (100 cm. x 10 cm.) with a bed length of 90 cm. The temperature is maintained at 4° with a recirculating cooler. The solution is pumped at an upward flow rate of 40 ml./hr. on a constant flow pump (LKB "Perpex 10200" Peristaltic Pump). 10 Ml. fractions are constantly monitored at 280 μ on a UV absorptionmeter. The active fraction is collected after 820 ml. of eluate have passed.

The duration of the run is 30 hours and the total volume of active fraction is 190 ml. The active fractions are pooled, dialyzed for 48 hours at 4° against several changes of large volumes of distilled water, shell-frozen and lyophylized.

Example 2

Purification of Isolated CEA Containing Fraction by Block Electrophoresis

The block electrophoresis medium, Sephadex G-25 Fine, is swollen with water for 2 hours at 80° and washed by decantation with borate of pH 8.6 and ionic strength 0.05 and then suction filtered through a sintered glass disk.

A thick slurry of the gel is poured onto a Lucite block support of 61 cm. x 7.5 cm. x 1 cm. in dimensions and allowed to distribute itself evenly along the plate to a depth of 1 cm. The surface is then blotted with cotton gauze sponges until firm but not completely dry.

The block is then fitted with 3 mm. chromatography paper contacts (Whatman) all aligned in the same direction of flow of the paper. The block is then placed in the electrophoresis apparatus and allowed to equilibrate for 1 hour under the operating conditions of 400 volts, with a constant current of approximately 20 mA at 4°. A 1 cm. strip is then removed from the center of the block and mixed well with 60 mg. of dried CEA from Example 1 which was

previously dissolved in a minimal amount of 0.05M borate, approximately 0.5

ml. The resulting slurry is then poured back in the central strip. One to

two drops of ferritin (6X recrystallized) at a concentration of 100 mg./ml. is

then spotted at the cathodal extremity of the block. 24 Hours after the start

of the run, the ferritin marker moves 18 cm. anodally. At that time the block

is removed from the electrophoresis apparatus and 2 centimeter strips between

the zone of application and the anodal extremity are eluted with 2M NaCl passed

through 0.20 µ disposable grid membrane (Nalgene*). The activity is localized

10-14 cm. anodal to the application zone with weaker activity being found 8-10

10   cm. anodal to the application zone.

Each active strip is eluted and dialyzed in pre-soaked 1-3/16" dia-

lyzer tubing against numerous changes of large volumes of distilled water at

4° for 48 hours. The dialyzate is shell-frozen and lyophylized to dryness.

The dried, purified CEA is stored under vacuum over calcium chloride at 4°.

The product has the following identifying characteristics:

It forms a single line precipitate with unabsorbed antiserum in gel

diffusion tests, is soluble in perchloric acid, it migrates anodally 10-14 cm.

in block electrophoresis at the same time a ferritin marker migrates 18 cm.

anodally when a borate buffer of pH 8.6 and ionic strength of 0.05 and 400

20   volts and 20 mA are the conditions of the block electrophoresis. Further, the

product has a molecular weight of 200,000 and a spectrophotometer absorption

peak wave length of 280 mµ.

Example 3

Preparation of Anti-CEA Antiserum

Twelve adult, male, New Zealand white rabbits weighing 2.0 kg. were

divided into 3 groups of 4 each. The different groups were immunized with

the following materials: normal colon tissue extract, tumor tissue extract

from the same individual from whom the normal tissue extract was obtained,

pooled human plasma. The tumor tissue extract was from specimens of tumors

30   diagnosed and confirmed to be adenocarcinoma of the colon. The normal tissue

extracts and tumor tissue extracts were formed in accordance with the proce-

dure described in Example 1. The pooled human plasma was obtained from 30

* Trade Mark

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normal donors representing all major blood groups.

The injections which were given twice weekly for 4 weeks contained 3 mg. of protein in 0.6 ml. of tissue extract or plasma emulsified in an equal volume of Freund's adjuvant (complete). Injections of 0.1 to 0.2 ml. were given into a foot-pad and the remainder intramuscularly into the flank. Twelve days following the last injections, the animals were bled from their marginal ear veins and the sera obtained from each group was pooled separately for testing.

The sera from each group was absorbed with normal tissue and the re-
10 sulting precipitate separated. The supernatants were then subjected to the Ouchterlony technique of double diffusion in agar gel. This was performed in one per cent agar-in-saline with merthiolate added as a preservative to a final concentration of 1/10,000. The patterns were cut in the gel plates so that the central and peripheral wells were spaced 1.0 cm. apart. Each well was filled with 0.15 ml. of test material. The antigen concentration used was 10 mg. protein per ml. Initial incubation of the plates was carried out in a moist environment at 37° for 24 hours to encourage diffusion of material from the wells. Patterns were allowed to develop in the same humid atmos-phere at 25° for seven days. The patterns from the serum of the animals which
20 were immunized with tumor tissue extract were a single line, indicating the serum contained antibodies specific for the tumor. No patterns developed from the sera of animals immunized with normal tissue or normal human plasma.

<u>Example 4</u>

Radioiodination of CEA

A 500 µl. reaction mixture is formed containing 100 µg. of Chlora-mine T, 250 µg. of CEA from Example 2 and 4 mCi of I^{125} in the form of KI. The reaction is allowed to proceed for 1 minute at room temperature and is then stopped by the addition of 240 µg. of sodium metabisulfite. The result-ing product I^{125}-CEA is separated from residual unreacted I^{125} by chromato-
30 graphy on a Sephadex G-100 column. The product has a specific activity of about 5 mµCi/µg.

Example 5

Preparation of Blood Samples for Radioimmunoassay

5 Ml. of 2.0M perchloric acid is added to each of 5 ml. of serum obtained from blood samples from 200 human subjects and stirred at room temperature for 20 minutes. The resulting precipitate is removed by centrifugation in a fixed-angle rotor at 9000 gravity for 10 minutes at 4°C. The resulting sediment is discarded and the supernatant dialyzed against cold, running tap water for 24 hours, and then against repeated changes of distilled water at 4°C. for an additional 24 hours. The dialyzed residue is then lyophylized to a dry powder.

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Example 6

Radioimmunoassay of CEA

During this procedure, normal human serum is diluted 1:100 with borate buffer (ionic strength 0.1, pH 8.4) and these preparations employed as diluents for the anti-CEA antiserum prepared from goats in accordance with the procedure of Example 3 and the I^{125}-CEA prepared according to the procedure of Example 4.

(a) Preparation of Standard Inhibition Curve

5 Ml. of standard CEA are added to a series of tubes, each of which contains the powdered extract of 5 ml. of normal human serum prepared according to the procedure of Example 5. A 500 μl. of diluted anti-CEA antiserum

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is then added to each tube. The resulting solutions are then incubated at 4° for 18 hours following which 500 μl. of I^{125}-CEA is added to each tube. The incubation is then continued for an additional 2 hours at 37°. Then 1.0 ml. of cold (4°) saturated aqueous ammonium sulfate is added to each tube. In the resulting 50 percent-saturated solutions of ammonium sulfate, the antibody-bound CEA undergoes coprecipitation, while the free CEA remains in solution. After a wash in 3.0 ml. of 50 percent saturated aqueous ammonium sulfate, at 4°, the I^{125}-CEA content of the precipitate is determined in a Nuclear-Chicago Dual Channel Automated Scintillation Gamma-Ray analyzer. The resulting stan-

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dard inhibition curve is as follows:
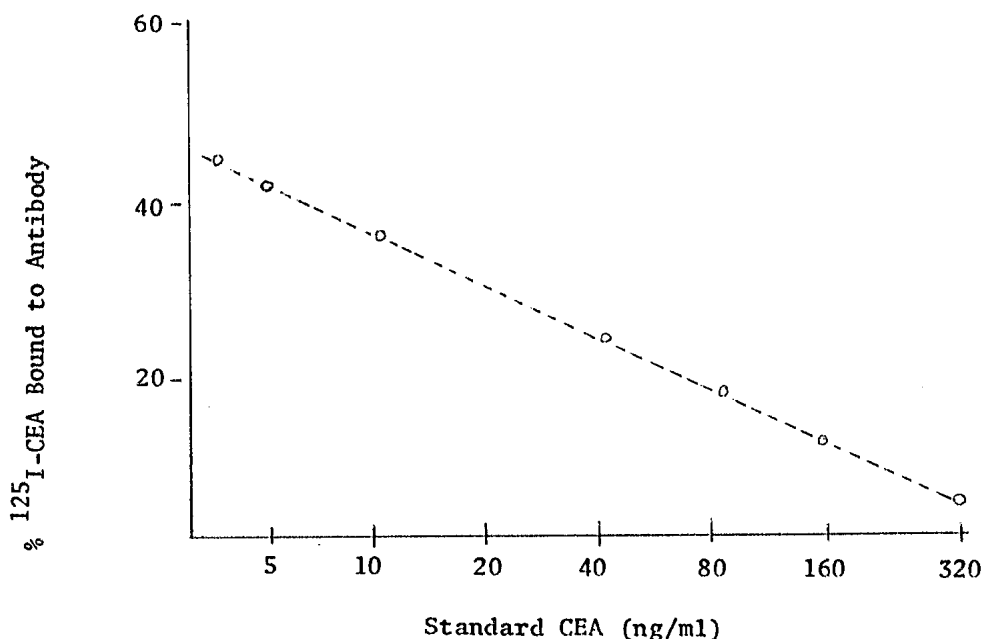
(b)   Radioimmunoassay of Serum

The powdered extracts of serum obtained from each of the 200 human subjects are processed in an identical fashion with the exception that no standard CEA is added to any of the specimens.

The results of the radioimmunoassay show that CEA could not be detected in any serum obtained from normal subjects, pregnant women, patients with cancerous diseases of non-digestive system organs, patients with non-malignant diseases of the digestive organs and patients with non-enteric, non-neoplastic conditions.

In 36 patients with adenocarcinoma of either the colon or rectum, studied at a time when tumor tissue was known to be present within the body, all but one serum was found to have detectable levels of circulating CEA. The only known false negative result recorded to date was in a patient with a localized, invasive polypoid adenocarcinoma of the transverse colon which prevented the CEA from entering the circulatory system. At operation, this patient was found to have a volvulus of the large bowel with circulatory impairment.

The lowest positive value was recorded in the serum of a patient with a malignant large bowel tumor, with 3 ng./ml. No false positive results were

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obtained. Patients who had undergone surgery for colonic or rectal cancer and who showed no evidence of residual or recurrent tumor growth did not have detectable circulating CEA. In seven cases where both pre-operative and post-operative blood samples were obtained, the post-operative values of serum CEA remained elevated only in the one patient with evidence of tumor dissemination in operation.

THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE
PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:

1.   A method for detecting the presence of carcinoembryonic antigen in blood which comprises (a) adding a measured amount of anti-CEA antiserum to a sample of perchloric acid extracted blood serum; (b) incubating the mixture; (c) adding a measured amount of radioactive tagged carcinoembryonic antigen to the incubated mixture; (d) incubating the mixture; (e) adding a protein precipitant to the incubated mixture thereby coprecipitating all the CEA-anti-CEA complexes; and (f) measuring the radioactive content of the supernatant or precipitate.

2.   The method of claim 1 wherein in step (a) the anti-CEA antiserum is diluted with normal blood serum to a dilution of 1:100 in a buffer of about pH 8.4.

3.   The method of claim 1 wherein in step (c) the CEA is tagged with I^{125}.

4.   The method of claim 1 wherein in step (e) the unreacted CEA remains in solution and the protein precipitant is saturated aqueous ammonium sulfate.

5.   The method of claim 1 wherein in step (f) the radioactive content of the CEA in the blood is determined by comparing to a standard curve.

SUBSTITUTE

REMPLACEMENT

SECTION is not Present

Cette Section est Absente