An Antiserum to Ovarian Mucinous Cyst Fluid with Colon Cancer Specificity

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SUMMARY

Antisera to mucin of benign mucinous cystadenomas of human ovary were produced in rabbits. These antisera were reacted with cancerous and normal tissue extracts by the Ouchterlony double diffusion test. Distinctive precipitin reactions occurred with colon cancer mucosal extracts. These bands were distinctive for colon cancer and could not be removed by absorption with normal colon mucosa from the same patient. Other mucin-producing tumors, such as stomach, lung, ovary, and breast, failed to show any definitive cancer precipitin with this antibody. It is postulated that the ovarian mucins contain antigenic material common to altered mucin in colon cancer.

INTRODUCTION

Attempts to demonstrate tumor-specific antibodies to human carcinomata have appeared frequently in the literature, and recently successful experiments indicate that this goal is being realized (1, 2, 6, 10). The difficulty in the past has been the segregation of normal tissue antigens from abnormal cancer antigens and the actual technical demonstration that these cancer antigens are specific (5, 7, 8).

Our work in this field has grown out of a project which has shown that blood group mucopolysaccharides are somehow related to distant cancer metastases (4). Since mucinous cystadenomas of the ovary contain very concentrated mucopolysaccharides, it was decided to explore the specificities of an antiserum that this material might produce in rabbits. The next logical step, of course, would be to investigate the antigen specificity of those human tumors known to produce mucin. Thus, cancer of the stomach, colon, ovary, and lung came under our experimental scrutiny. We here report the immunologic results obtained in studying the reactions of antimucinous cyst fluid rabbit sera against extracts of adenocarcinomata of the colon in humans.

MATERIALS AND METHODS

Mucinous Cyst Fluid Immunizing Material. Seven mucinous ovarian cysts (formerly referred to as pseudomucinous cystadenomas) received from surgery were aspirated under sterile conditions and stored in the refrigerator at 4°C. The titer of blood group A and/or B and H substance was determined and electrophoresis of the proteins and glycoproteins performed. The cyst fluids contained relatively little protein according to electrophoretic protein staining and the biuret reaction. The cyst fluids gave strong periodic acid-Schiff reactions.

Preparation of Antimucinous Cyst Fluid Rabbit Sera. Twenty New Zealand white, virgin female rabbits, weighing four to five pounds each, were immunized in one group. Seven groups in all were used. A preimmunization blood sample was obtained as a control. Immunization consisted of fourteen 1-ml intraperitoneal injections of cyst fluid on a biweekly basis. Occasionally, the cyst fluids required homogenization or dilution with saline in order to render them sufficiently fluid for injection. The immunized rabbits were bled one week after the 14th injection, with further bleedings dependent on the quality of the serum obtained. The first bleeding of the rabbits was the best, and further immunization only seemed to produce a diffuse and weaker reaction. We usually pooled the first and second bleedings which were drawn one week apart to make our final antimucinous cyst fluid rabbit serum reagent. One or two rabbits from each series of 20 animals would produce satisfactory anti-cyst fluid serum which would be distinctly reactive with colon cancer extracts. When a serum was found to react satisfactorily to colon cancer extracts, dilutions were carried out on the diffusion plates and a titer of 1:8 diluted in normal rabbit serum could be obtained.

Preparation of Extracts. At least five grams of histologically proven colon cancer tissue were obtained from a central area of the tumor. A portion of normal colon mucosa was removed from a site at least five or ten centimeters from the pathologic area. Effort was made to avoid muscle tissue, but in some samples of normal colon it was deliberately included. All samples were frozen until extracted. Extraction consisted of ho-
mogenization with either a semimicro or micro Eberbach attachment for the Waring blender. A volume of normal saline solution (N/SS) equal to double the weight of the sample was added to facilitate homogenization. The coarse fibers were separated by centrifugation, and the lipid material was removed from the supernatant with a spatula. The tissue extracts were filtered through Whatman No. 1 filter paper and stored in the frozen state. Table 1 gives a summary of the extracted tissues and antisera employed.

Bacterial cultures of \textit{Escherichia coli}, \textit{Klebsiella pneumoniae}, and \textit{Aerobacter aerogenes} were extracted with potassium hydroxide to obtain polysaccharides. These extracts were used for antisera absorption in the agar plates.

The colon cancer extract produced another moderately intense precipitin band which crossed over the cyst fluid band, indicating nonidentity.

Most of the bands seen in Chart 1 could be removed by serum absorption, indicating that the cyst fluid secretion is composed of many components contained in serum. Pretreatment of the agar with human serum (Chart 2) eliminated these bands, leaving the bands concerned with mucosa and cancer tissue as well as the cyst fluid. All precipitin bands could be eliminated by a gel pretreated with cyst fluid.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|}
\hline
Tissue & Normal tissue & Aqueous cancer extracts & Antisera no. employed \\
\hline
Colon & 28 & 28 & 3, 7, 13, 14, 17 \\
Stomach & 7 & 7 & 13, 14 \\
Lung & 5 & 5 & 17 \\
Breast & 3 & 10 & 14, 17 \\
Ovary & 1 & 5 & 14 \\
Kidney & 3 & 3 & 14 \\
\hline
\end{tabular}
\caption{Tissue extracted and rabbit antisera number employed in the gel diffusion studies.}
\end{table}

Preparation of Ouchterlony Double Diffusion Agar Plates.

Two types of agar were used: basic 1% agar and treated agar. The treated agar consisted of basic 1% agar with 30\% pooled human serum by volume. Various concentrations of cyst fluid, saliva, and bacterial or tissue extracts were also added to the treated agar.

Basic agar was prepared by heating 1 gm of Difco Noble agar in 100 ml of buffered normal saline, pH 7.3. Agar treated with serum alone was prepared by dissolving 1 gm of agar in 70 ml of buffered saline. The heated agar was cooled to 70\(^\circ\)C and 30 ml of pooled human serum prewarmed to 40\(^\circ\)C were added. Other treated agars were prepared by dissolving 1 gm of agar in 60 ml of buffered saline. After the agar cooled to 70\(^\circ\)C, 30 ml of pooled human serum and 10 ml of tissue or bacterial extract, saliva, or cyst fluid were added. Then 16 ml of each type of agar were poured into a separate scratch-free plastic Petri plate and allowed to cool.

Diffusion wells were punched in the plate in various patterns using a No. 4 corkbore of 8 mm diameter for antisera and a No. 3 (6 mm) for tissue extracts. The distance between the centers of the diffusion wells was 15 mm. Extracts and antisera were inserted into their respective wells with a Pasteur pipet until visually level with the agar surface. Incubation was at room temperature in a moist chamber. The plates were observed and the reaction recorded for a two-week period.

RESULTS

The mucinous cyst fluid antisera (anti PS 14) produced many precipitin bands with the tissue antigens (Chart 1). The antisera produced a single intense band with the cyst fluid.
Chart 3. Ouchterlony reaction of native polyvinylpyrrolidone-concentrated, and blood group substance-absorbed mucinous cyst fluid antisera to colon cancer extract.

Chart 4. Ouchterlony reaction of mucinous cyst fluid antisera to cyst fluid and normal and cancer colon extracts in serum-treated and in serum and secretory saliva-treated agar.
The number of precipitin bands to colon cancer extracts varied with the different antisera and extracts. Two of the antisera produced exhibited single reaction bands (Chart 2). However, after concentrating one of these antisera with 10% polyvinylpyrrolidone, a second band was visible (Chart 3). Other antisera gave two or three bands to colon cancer extracts (Charts 5–7).

In order to eliminate the possibility that some of the antibody components might be related to A, B, or H antibodies, A and B secretor salivas were added to the agar plates. The antisera were also absorbed with human red cells of groups A, B, and O. This treatment did not alter the reaction of the antibody to cyst fluid or cancer extracts (see Charts 3, 4).

Bacterial extracts, prepared as previously described, were also used to treat the gel diffusion plates to make sure that bacterial products were not contaminating the colon extracts and causing the precipitation reaction observed. None of these extracts inhibited the cancer or normal colon reactions observed.

The general reaction pattern of mucinous cyst fluid antisera (anti PS 14) produced many precipitin bands with the tissue antigens (Chart 1). The antisera produced a single intense band with the cyst fluid. The colon cancer extract produced another moderately intense precipitin band which crossed over the cyst fluid band indicating nonidentity.

The general reaction of mucinous cyst fluid antisera with colon cancers and their respective normal mucosa extracts is shown in Chart 5. It will be observed that in each instance there is a distinct inner band at the cancer wells not present at

Plate 339: 1% AGAR PRETREATED WITH 30% HUMAN SERUM

Well #1 Anti-PS H-14 (Equal Part 1st and 2nd Bleedings)  
Well #2 N/SS Ext. Normal Colon (A.)  
Well #3 Anti-PS H-17 (Equal Part 1st and 2nd Bleedings)  
Well #4 N/SS Ext. Cancer colon (A.)

Chart 6. Ouchterlony reaction of two mucinous cyst fluid antisera to normal and cancer colon extracts in human serum-treated agar.

Plate 343: 1% AGAR PRETREATED WITH 30% HUMAN SERUM AND 10% N/SS EXT. NORMAL COLON (A.)

Center Well Anti PS H-14 (Equal Parts 1 and 2 Bleedings)  
Well #1 Anti-PS H-14 (Equal Part 1st and 2nd Bleedings)  
Well #2 N/SS Ext. Normal Colon (A.)  
Well #3 Anti-PS H-17 (Equal Part 1st and 2nd Bleedings)  
Well #4 N/SS Ext. Cancer colon (A.)

Chart 7. Ouchterlony reaction of two mucinous cyst fluid antisera to normal and cancer colon extracts in human serum and normal colon extract-treated agar.
The specificity of the antisera for colon cancer tissue is further illustrated in Charts 6 and 7 which show the results of Ouchterlony plates pretreated with human serum and normal colon extracts. The treatment with normal colon extract excludes the normal colon band, leaving the cancer precipitin bands. Conversely, when the plate is treated with cancer extract, the cancer band is eliminated, leaving the normal colon band. Cancer bands of wells 1 and 5 show a distinct crossing-over of normal colon bands indicating non-identity between the two.

The specificity of the antisera for colon cancer tissue is further illustrated in Charts 6 and 7 which show the results of Ouchterlony plates pretreated with human serum and normal colon extracts. The treatment with normal colon extract excludes the normal colon band, leaving the cancer precipitin bands. Conversely, when the plate is treated with cancer extract, the cancer band is eliminated, leaving the normal colon precipitin unaltered (compare Chart 8 with Chart 5). These procedures seem to offer evidence that the antigens of adenocarcinoma of the colon and normal mucosa may be demonstrated as separate substances by antisera produced in rabbits immunized with ovarian cyst mucins. Of the 28 adenocarcinomatous extracts, 13 gave the discrete precipitin band illustrated while 6 more showed probable reactions, thus indicating a relatively common association.

DIscUSSION

Whitebsky et al. (7, 9) have reported that globulins extracted from the ovary and intestinal tract are “surprisingly specific” antigens for the production of antisera in rabbits. Furthermore, two of the most potent antisera they produced were from pseudomucinous cystadenomas of the ovary. He speculated that the antisera produced might even be able to recognize ovarian tumor histologic types. Our results herein reported seem to corroborate his findings that, indeed, unaltered ovarian mucoproteins are antigenic and that, in addition, the antibodies produced will frequently recognize an antigen of adenocarcinoma of the colon.

Gold’s (1) work is similar to ours in that he compared both normal colon mucosa and cancer tissue extracts and was able to detect two cancer determinants not present in the normal mucosa. He could not be sure, as we cannot be sure, that a quantitative difference is entirely ruled out. However, in the range of dilution of the antisera used, cancer antigens were detected and normal tissues were negative. Absorption techniques were employed.

Hakomori (3), another investigator working with colon cancer tissue, purified a large cancer mass by column chromatography and chemical means to produce an antigen capable of producing titers of antibody to colon adenocarcinoma. He reports his antigen to be a glycolipid not unlike blood group substances. An exhaustive study using this antibody to identify tumors has not yet appeared.

The antisera we have produced with ovarian mucin has the following activity: (a) it produces precipitin bands with cancers of the colon not detected with normal colon mucosa; (b) cancer extracts absorb the antisera components responsible for the precipitin bands which recognize colon cancer; (c) ovarian mucin from which the antisera was produced also absorbs all of the cancer antisera reactants; (d) blood group substances and normal colon mucosa do not absorb the components of the antisera responsible for the colon cancer reaction; (e) the failure to reeicit a good antisera response after the mucin had been frozen 3 months suggests a labile nature of the antigen; (f) ovarian mucinous cystadenomas differ in their ability to produce a satisfactory colon cancer antibody, and generally the more thickly mucinous fluids have yielded the best reacting sera; (g) other adenocarcinomas of stomach, ovary, lung, and breast have not given distinctive precipitin bands by gel diffusion methods using the ovarian mucin antisera.

Evidence is herein added to the mounting accumulation of data that cancers in humans contain antigenic determinants differing from the normal antigens of the same organ. Our evidence suggests that, in the altered process of colon cancer metabolism, an antigenic substance common to mucinous cystadenoma of the ovary is produced. This substance is not present in detectable amounts in normal colon mucosa by the various concentration dilution and absorption techniques used in the procedures herein reported.

REFERENCES

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