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Abstract

From the data presented in this communication, it might be concluded that a cancer specific substance, which can be demonstrated in gel diffusion, is present in human cancer tissue, common to various epithelial cancers of different individuals, although it may vary in its concentration. Needless to say, this substance is quite different from the so-called interspecies antigen or organ specific antigen, as proved by the present experiments. Furthermore, this substance can be eluted well by the Fluorocarbon treatment and it displays physically and chemically unstable characteristics. This substance is likely to be included in the microsome fraction and soluble fraction which was determined by gel diffusion technique. However, the association of this substance with other specific antigenic substances of human cancer, concerned with “delayed type skin reaction”, ”cytopathogenic antiserum against cancer cell”, and ”complement fixing antibody in serum of patients with cancer”, has not been elucidated in this study.

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IMMUNOCHEMICAL ANALYSIS OF ANTIGENIC PROPERTIES OF HUMAN STOMACH CANCER

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Many investigators have attempted to demonstrate specific tumor antigen of human cancer, which is not present in normal cells. Available works have[17,29,36] so far revealed no qualitative nor constant quantitative differences between cancer tissue and comparable normal cells. However, the clinical observations of the phenomena such as spontaneous remissions of cancer[7,8,19], plasma cell reaction in breast cancer[1] (inflammatory infiltration in cancer) or some positive skin reactions with homologous tumor antigen in patient with inflammatory cancer[16], seem to sustain the working hypothesis that man indeed has a host defence mechanism against cancer. Thus, if some specific antigen is found in cancer tissue, it may be assumed that "cancer" can be detected in early stage, which can be treated and prevented with utilization of specific immune mechanism. The implications of immunology for the problems of clinical cancer are theoretically of significance and practically of great use. Therefore, in the present study attempts have been made to demonstrate the nature of specific antigenicity of human stomach cancer by rigorous methods of analytical chemistry, mainly by means of gel-diffusion technique. At present, there is a number of reports[2,36] related to the antigenicity of human stomach cancer, examined by the method of gel-diffusion, though the results are not always identical in every case. Moreover, it is not quite certain whether any specific cancer antigen does exist. Some authors[24,32] have shown the loss of organ specificity in human cancer, while others[2,17,35,36] have successfully demonstrated the presence of specific cancer antigen.

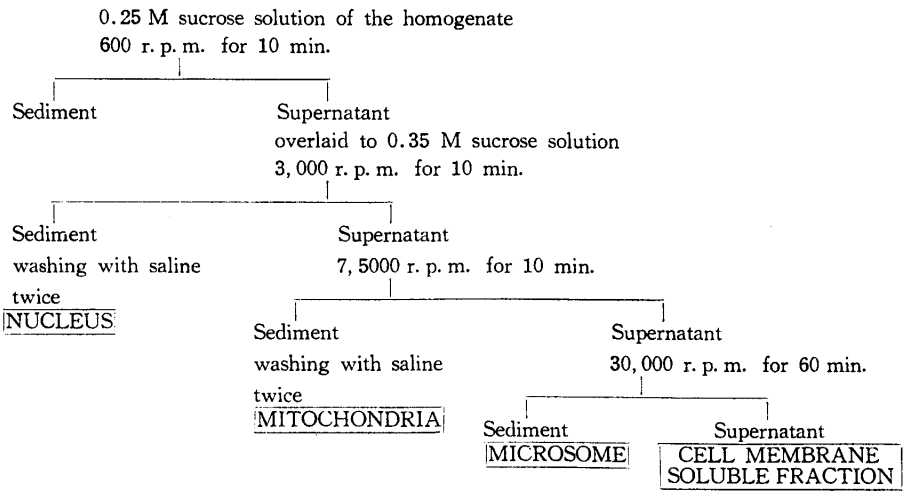
The present investigation is concerned with a demonstration of antigenic components specific to human cancer tissue. With a view to detect such specific antigenic components, rabbits were immunized with extracts from human stomach cancer tissue. As immnne sera of rabbits might contain multiple antibodies against both the normal and the tumor tissue proteins, attempts were made to differentiate these antibodies by the absorption with various kinds of human tissues and tumors. Further, in order to detect and characterize the cancer specific antigenic components some immunological observations were carried out. As the result a cancer specific property was detected in the human

stomach cancer extract which was a moderately soluble, physically and chemically unstable sudstance, and could be eluted from the human cancer tissue extract by the Fluorocarbon treatment without any loss of pertinent antigenicity.

MATERIALS AND METHODS

Preparation of Antigens: The antigen was obtained from cancerous tissue, freed from necrotic portion, of the fresh specimens of human stomach cancer removed at operation; 23 cancer specimens were pooled and kept frozen at −40°C in the refrigerator until used. Normal stomach mucosa of the same number, some specimens of various organ cancers and normal organs were collected and similarly treated. The tissues were rinsed with chilled, buffered physiological saline (pH 7.5) and homogenized for 10 minutes in Waring-blender and Potter's homogenizer at 4°C. Further cell disruption was obtained by two to three minutes' treatment with a ultrasonic disintegrator. Sucrose was added to the suspension to make a 0.25 M solution and the cellular components were separated by the differential centrifugation; after the centrifugation at 1,000 r. p. m. for 10 minutes, the supernatant was used as the basic material, designated crude cancer extract (CE) for immunization of rabbits in this study. Further cell fractionation was carried out according to LITTERFIELD's method (Table 1).

Table 1 Differential Centrifugation Analysis of Human Stomach Cancer Homogenate According to Litterfield's Method



Preparation of Antiserum: The antiserum was prepared in six rabbits (2.5～3 kg) by two subcutaneous injections of the antigen-Freund's[10] adjuvant emulsion and intravenous injections were repeated at intervals of two～three days over 10 weeks. The mixture of 2 ml of mineral oil, 0.5 ml of Arlacel A,

and 20 mg of dried, heat-killed mycobacteria, was thoroughly stirred, and to the mixture 2 ml of crude cancer extract were added drop by drop, emulsified thoroughly after each addition. In order to avoid the coagulation of the blood, in the case of the intravenous injection, this emulsion was diluted ten-fold, mixed with heparin (500 I. U./ml antigen solution) and given slowly 10 ml of it to the rabbits at least for 10 min. The total amount of nitrogen in the antigen given to each rabbit was about 25 mg. The animals were periodically bled every seventh day, after the precipitin titer of each blood was estimated.

The inoculation procedures are shown in Table 2. The serum was stored by freezing at −40°C, with addition of merthiolate to give a final concentration of 1 : 10,000.

Table 2 Immunization Procedure & Precipitate Reaction of Immune Rabbit Serum against Human Stomach Cancer Extract

Day	Human stomach cancer extract mg N	Route of Injection	Volume of Bleeding & Accidents	Precipitin Reaction of Serum
1	4	I. M. (F. A)	10 ml	(control)
3	4	I. M. (F. A)		
9	4	I. V.	10 ml	(−)
11	2	I. V.	No. 5 (*)	
17	2	I. V.		
18	3	I. V.		
31	4	I. V.	10 ml	(−)
45	0.5	I. V.	10 ml	(±)···(−)
47	0.5	I. V.		
48	1.0	I. V.		
61	0.6	I. V.	10 ml	(+)···(±)···(−)
62	1.0	I. V.		
69			10 ml	(++)···(+)···(±)
88			20 ml	(++)···(+)
95			20ml No. 6 (*)	(++)···(+)
105			20 ml	(++)···(+)
116			20 ml	(++)···(+)

(*) = expired (++): remarkable (+): moderate (−): scarce

Ring Test: For the detection of precipitin antibody against human stomach cancer extract, the ring test is carried out. The rabbit antiserum was made transparent by centrifugation at 3,000 r.p.m. for 30 min. This clear serum was introduced into tubes (3 × 50 mm) and very carefully overlaid with an appropriately diluted antigen solution. The serial ten-fold dilutions of the antiserum were set for estimation of the precipitin titer of this system, and placed in consecutive order with a saline control. A positive reaction, which indicates visible precipi-

tation of the antigen-antibody complex, is observed as a turbidity ring at the interface of these solutions. This reaction occurs within a few minutes, if the precipitation amounts at least to 1.0 γg of protein.

Estimation of Protein (Kjeldahl Determination for Nitrogen): The estimation of the protein either in tissue extracts or precipitates, was performed according to the Micro-Kjeldahl method, modified by F. C. KOCH and T. L. MCMEEKIN[21].

Quantitative Determination of Equivalence Zone: The point of maximal precipitation lies close to the equivalence zone in general. To determine the equivalence zone of this system, precipitation titration was performed in a procedure in which the amount of the antigen may vary and the amount of the antiserum kept constant. The estimation of nitrogen in the course of this study was made according to the modified Micro-Kjeldahl method as already mentioned.

Paper Electrophoresis[27]: In the course of immunization of rabbits with crude cancer extract, as previously mentioned, the paper electrophoresis of the antiserum was regularly carried out for detecting the increased γ-globulin fraction in the antiserum.

For the buffer solution of this electrophoresis, barbital buffer (pH 8.6 = 0.01) was applied. No. 51 filter paper made by Toyoroshi Co. (Japan) was used in this study. The apparatus for the paper electrophoresis was the Dobayashi model of Natsume make. The electrode vessels were filled with 2% KCI solution and the bridges with 3% suspention of agar in 2% KCI. The electrophoresis was run for 4 hours at a constant voltage of 250, with average current of 5 mA at room temperature. After being dried in an electric desiccator at 110°C for 10 min the papers were stained in 10% staining solution of Amidoschwalz 10 B for 20 min. Then, decoloration was done in 2.0% acetic acid solution, drying in the desiccator, and soaked in melted paraffin. Thereafter, the papers were set in the integral densitometer of Atago make for the calculation of each fraction of the serum protein.

Starch Block Electrophoresis: For the purpose of purifying the anticancer serum of nonspecific antibodies or other components according to the method of BLOEMENDAL[5], starch block electrophoresis was performed. The antiserum of 10ml of 50～60 mg/ml protein concentration was introduced into the block, 10 cm wide and 40 cm long. The electrophoresis was run for 24 hours at a constant voltage 400 with current of 50～60 mA at 4°C. Each eluate from the segment 1 cm of the block was centrifuged at 3,000 r.p.m. for 30 min. With the clear suprnatant, the ring test was performed to determine the presence of specific precipitin antibody.

Gel-diffusion: The plate method of OUCHTERLONY[25] was applied for the

analysis and identification of the various components, contained in the cancer extract by means of the rabbit immuneserum prepared against it. Agar solution was prepared in the following manner ; 50 ml of phosphate buffer solution (pH 7.2, 0.1 M), 50 ml of Merthiolate (1 : 1,000) and 400 ml of distilled water were added to 500 g of 4 % agar which had been melted and solidified previously, to make 2 % agar solution.

The agar thus prepared (8 ml) was pipetted into the petri dish 10 cm in diameter. After solidification of the agar, 15 ml of agar were superimposed by pipetting the top agar layer to form the wells in a proper arrangement[22]. The rabbit anti-human cancer serum (0.25 ml) was placed in the center well. Each sample, 0.25 ml of the antigen solution, various crude tissue extracts or the extracts Fluorocarbon-treated, was placed in the circumferential wells. The precipitation was allowed to take place by storing the plate in a humidified 37°C incubator, empirically set 3 to 7 days. Normal human serum, extract from stomach mucosa homogenate and extract from liver homogenate were used for the absorption of the nonspecific antibodies contained in the rabbit immuneserum against the cancer extract. The extract from cancer of other organs such as colon, rectum, breast, lung, brain, thyroid, was applied for further evaluation in order to compare with the antiserum against the stomach cancer extract for the purpose to elucidate the characteristics of cancer specific antigen. On the basis of interference phenomena in the zone of precipitation, it is possible to classify the immune reactions as "reaction of identity", "reaction of nonidentity" and "reaction of partial identity"[33]. Judging from the precipitin band developed on the plate, it is possible to tell whether the antigens and the antibodies being compared are identical, distinct or cross-reacting. All the tests were performed in triplicate in each experiment.

Fluorocarbon Treatment[11] *on Antigen* : This crude cancer extract itself is not necessarily soluble in saline. For the studies of the antigen-antibody reaction by means of gel-diffusion it is necessary to have the antigenic fraction in a diffusible form. For the purification of the crude cancer extracts, the extract was treated with Fluorocarbon* in the following manner.

To the crude extract the same volume of Fluorocarbon was added. The mixture was blended thoroughly in a stirring apparatus for 2～3 min with interval of a few minutes until the formation of white foam in the mixture. The resulting gelatinous mass was centrifuged at 3,000 r. p. m. for 15 min to isolate the insoluble substance like cell debris. After repeating this procedure three times, finally the supernatant turned transparent and its protein content was reduced to one-fourth that of the original crude extract. The Fluorocarbon treated antigen was contained in the aqueous supernate. With this solution the gel-diffusion test was carried out. Fluorocarbon* is trichlorotrifluoroethane

(CCl_2F-$CClF_2$), having a boiling point of 45.57°C and a melting point of −35°C which is a colorless transparent liquid, manufactured by the Osaka Kinzoku (Japan).

Tissue Sediment for Absorption Procedure: The various tissue sediments were prepared according to the Coon's method[6] and were used for absorption of the nonspecific precipitin antibodies present in Anti-CE rabbit serum.

RESULTS

Preparation of Rabbit Antiserum Against Stomach Cancer Extract: Through a preliminary study with the cancer extract, it was clarified that two subcutaneous injections of the antigen-adjuvant emulsion were not enough inoculation for the rabbit to produce a suitable antiserum against the crude tissue extract like the stomach cancer tissue. Therefore, a combination of Freund's adjuvant method and the intravenous method was employed for the preparation of the rabbit antiserum in this study. In the case of the inoculation with serum, it did not take more than 4 weeks for the rabbits to produce a favorable antiserum. However, in the present it took more than 6 weeks and it is thought that the period of 10 weeks is desirable for rabbits to produce a fully satisfactory antiserum to be used for the following immunochemical investigation. In the course of the inoculation of rabbits, blood was examined every tenth day for the purpose of detecting gradual variation in the serum protein fractions with lapse of time, especilly in γ-globulin fraction by means of the paper electrophoresis. A partial result is shown in Table 3. Gradual variation was observed

Table 3 Rabbit Serum Protein (inoculated by human stomach cancer extract) Fractionated by Paper Electrophoresis

Day of Bleeding	Albumin	Globulin			Precipitin
		α	β	γ	
control	42.4 %	16.5 %	14.4 %	26.6 %	(−)
20	44.3	13.4	10.0	32.0	(−)
40	44.0	11.3	9.0	35.3	(±)
70	29.0	10.1	4.3	56.5	(++)
90	35.9	11.9	6.8	45.3	(++)
100	37.8	9.0	7.0	46.1	(++)
120	41.2	11.0	8.2	39.6	(++)
140	42.0	11.6	10.0	36.4	(++)

for 70 days since the first injection. A remarkable change was observed on the examination of 70th day blood; a decrease in albumin fraction and β-globulin

fraction, and an increase in γ-globulin fraction, which were distinct from the data of the control serum. The antigen-adjuvant emulsion injected subcutaneously as well as the effect of the antigen injected intraveously might have appeared at this period. As for the precipitin reaction of this serum, it proved to be positive corresponding to the time when γ-globulin was found to have increased remarkably. The intravenous injection of the crude antigen was continued for a prolonged period as shown in Table 4, with the injections of 1.5 mg nitrogen in an average dose twice a week. The total of the antigen nitrogen amounts to

Table 4 Long-term Inoculation of Rabbits for Production of Anti-Human Cancer Serum

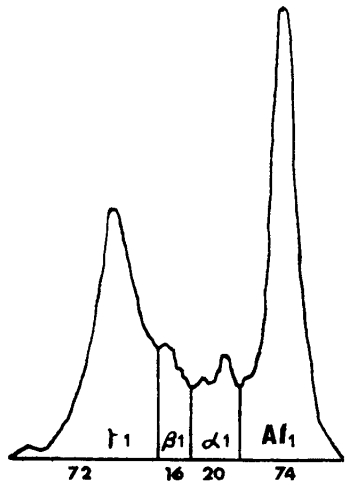
Day	Human Stomach Cancer Extract mg N	Route of Injection	Date of Bleeding
1	6	I. M. (F. A)	bleeding
2	3	I. V.	
3	3	I. V.	
10	1	I. V.	bleeding
11	2	I. V.	
12	2	I. V.	
18	0.5	I. V.	bleeding
19	1.0	I. V.	
20	2.0	I. V.	bleeding
30	1.0	I. V.	bleeding
40	1.0	I. V.	bleeding
41	1.5	I. V.	
42	1.5	I. V.	
43	1.5	I. V.	
51	0.5	I. V.	bleeding
52	1.0	I. V.	
53	1.0	I. V.	
60	0.2	I. V.	bleeding
61	0.6	I. V.	
62	1.0	I. V.	
72	1.5	I. V.	bleeding
80	0.5	I. V.	bleeding
81	0.5	I. V.	
82	0.5	I. V.	
90			bleeding
102	0.6	I. V.	bleeding
103	0.6	I. V.	bleeding
110			
111	0.6	I. V.	
120			bleeding
140			bleeding

about 25 mg by the time the antiserum against cancer extract developed adequately for the use in gel diffusion tests. With continuous, repeated injections of the immuneserum attained the state whereby it could be used for the period of more than 2～3 months after the initial injection. On the basis of the data of precipitin titration, the rabbit was bled by the puncture of an ear artery when the precipitin titer reached 1 : 500. The immunesera were stored, frozen in a refrigerator at −20°C, with the addition of merthiolate solution in the concentration of 1 : 10,000.

Absorption Procedures with Various Tissue Extracts: For the purpose of determining what proportion of the increased γ-globulin is especially affected by the immunization with the cancer extract, absorption tasts were conducted. A mixture of the antiserum and an appropriate amount (100 mg to 1 ml antiserum) of human liver powder was stirred in the hot water bath at 37°C for two hours, and then centrifuged at 30,000 r. p. m. for one hour. The supernatant was examined by paper electrophoresis and compared with unabsorbed original serum. The absorption of antiserum with human stomach mucosa sediment was performed in the same manner aforementioned, and likewise the absorption with stomach cancer sediment. The results of these trials are illustrated in Fig. 1. It is evident that at least 50% of increased γ-globulin in immuneserum depends on the precipitin antibody formed by the injections of crude stomach cancer extract. The precipitin antibody was considerably absorbed by the human stomach mucosa sediment, but moderately by the human liver sediment. These results suggest the existence of several antigenic properties in stomach cancer, common to those of stomach mucosa and liver. It may be concluded that the immune-system between the cancer extract and its immuneserum seems to be composed of a mixture of multiple antigens-antibodies. The rest of the increased γ-globulin, not absorbed even by the stomach cancer extract (homologous antigen), is suspected to be due to a mere increase in γ-globulin without any relation to the immunization or non-precipitin antibody, unabsorbed. It is presumed that there is much significance in the disparity between the datum (iv) of the experiment by the cancer sediment and by that (iii) of the normal mucosa sediment. Namely, it has not been clearly determined from these results whether this disparity depends upon mere quantitative difference or qualitative difference of the antigenic properties of these two antigens. Consequently, it is obscure whether the cancer specific antibody is included or not in this rabbit immuneserum. To clarify this point, the following three attempts were made.

At first, quantitative precipitation test of this system was performed for the purpose of determining quantitatively this sytem's equivalence zones and OP ratio to be appropriate for the absorption in further procedures. Secondly, from the aspect of "antiserum", analysis was done of the rabbit immuneserum in each

Fig. 1



(i) Paper electrophoresis of nonabsorbed original Anti-CE rabbit serum as a control

Amount of each γ-globulin absorbed by various tissue sediments is to be presented in per centum comparing with that of the original γ-globulin of the control and calculated from the formula blow.

After absorption of Anti-CE-serum by addition of human liver sediment (Al_2, 5_2):

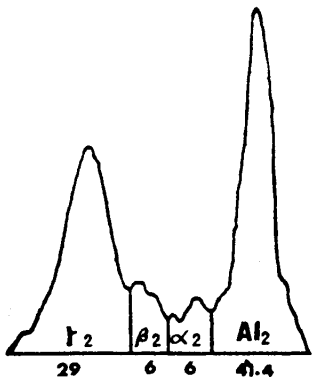
$$\left(1-\frac{\gamma_2 Al_1}{\gamma_1 Al_2}\right)\times 100=11\%$$

After absorption of Anti-CE-serum by addition of stomach cancer sediment (Al^3, γ^3,):

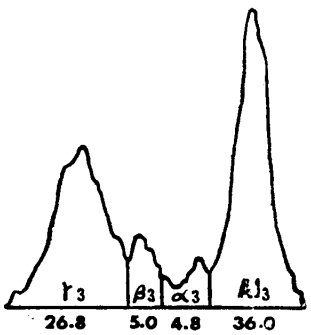
$$\left(1-\frac{\gamma_3 Al_1}{\gamma_1 Al_3}\right)\times 100=23\%$$

After absorption of Anti-CE-serum by addition of stomach mucosa sediment (Al_4, γ_4,):

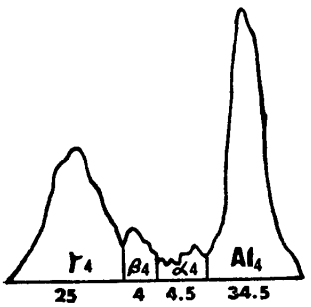
$$\left(1-\frac{\gamma_4 Al_1}{\gamma_1 Al_4}\right)\times 100=25\%$$



(ii) Paper electrophoresis of absorbed Anti-CE-serum by human liver sediment



(iii) Paper electrophoresis of absorbed Anti-CE-serum by stomach mucosa sediment



(iv) Paper electrophoresis of absorbed Anti-CE-serum by stomach cancer sediment

antibody to detect a cancer specific antibody. Thirdly, from the aspect of "antigen", analysis was carried out with these complex antigens contained in the stomach cancer tissue by means of the fractionation, differential centrifugation, DEAE cellulose column, and gel-diffusion tests, especially for the detection of the cancer specific antigenic properties of the stomach cancer extract.

Quantitative Precipitation Test of this Immune-system: When the curve is drawn with the results of quantitative precipitation test of this immune-system, it is of a mountain shape as shown in Fig. 2. In the spindle of this

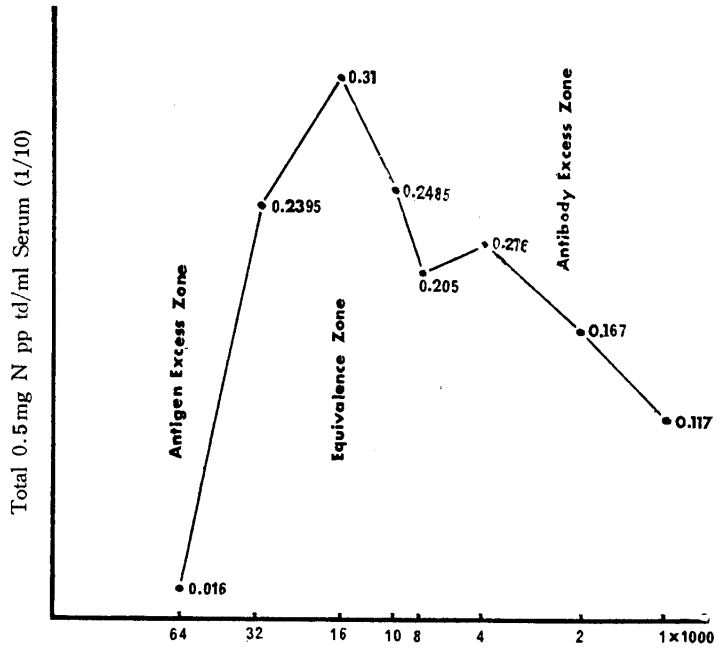


Fig. 2 Quantitative Precipitation Curve

graph, the precipitated protein from immuneserum is represented as the amount of mg nitrogen. In the axis of abscissa, the number of dilution of the introduced antigen solution is given. From this curve, it is obvious that the equivalence zone of this system is at 16-T antigen dilution. The proportion of the antigen to the antiserum at OP ratio is 0.4 mg nitrogen to one ml (10 mg nitrogen) serum. At this point this system may be divided into an antigen excess zone and an antibody excess zone. During this process the data obtained by the method of Micro-Kjeldahl, occasionally varied considerably on the estimation of nitrogen contents of the cancer extract, even with the same specimens. One source of the errors in this process seems to be due to the tissue extract like this being hardly digested and partially due to the lack of qualitative uniformity in the cancer extract.

Attempts of Purifying Antiserum by Starch Block Electrophoresis: Next, the analysis of antibody complex was performed by means of the starch block electrophoresis.

The rabbit immuneserum is known to have multiple antibodies, which correspond to antigenic properties of the homologous cancer extract. The starch block electrophoresis (Fig. 3) presented substantially similar results to these of

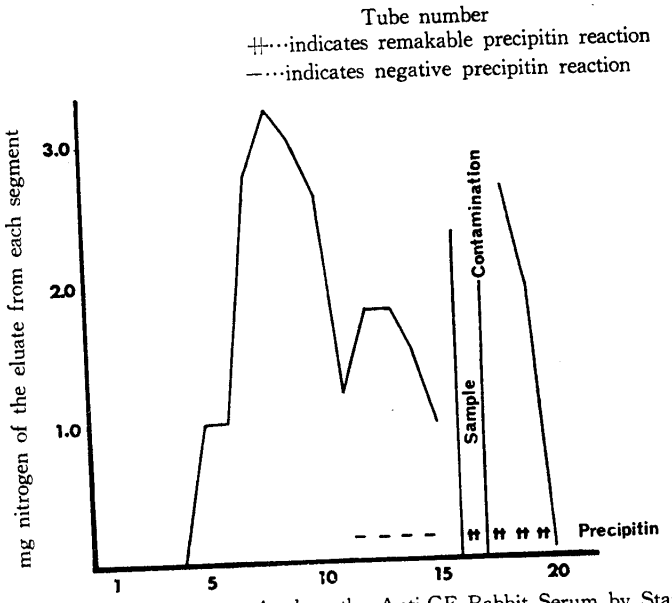


Fig. 3 An Attempt to Analyse the Anti-GE Rabbit Serum by Starch Block Electrophoresis

the paper electrophoresis previously observed. Samples No. 17, No. 18, No. 19, and No. 20, eluted from each segment of starch block cut 1 cm each were studied by immunological method to detect the cancer specific antibody by absorption with the tissue sediments of different kinds. The X-mark in the figure corresponds to the cancer specific reaction detected by ring test. It has also been clarified that both γ-globulin and β_2-globulin react with the homologous antigen. Consequently, it is concluded that the detection of any specific antibody reacting with the cancer extract is impossible. Other methods for purifying the immunesera were tried, such as DEAE cellululose column and Tisselius electrophoresis, but failed to obtain any definite results.

Analysis of the Antigenic Properties of the Cancer Extract by the Method of Ouchterlony: The gel-diffusion technique was employed in order to separate antigens and ascertain the antigenic properties in the cancer extract on the basis of differential diffusion through agar. The antiserum (Anti-CE) was then used as the serological reagent to identify these antigens as illustrated in Fig. 4. The figure illustrates the reactions of the rabbit antiserum (Anti-CE) with the homologous stomach cancer extract (CE) and normal stomach mucosa extract (ME). The reaction with the CE is shown by a converging arc consisted of 3～4 distinct lines, and that of the ME is shown by a similar arc, consisted of somewhat wider diffuse bands. The reaction of the antiserum with normal stomach mucosa extract is slightly reduced.

In this instance, the partial cross reaction can be observed between these

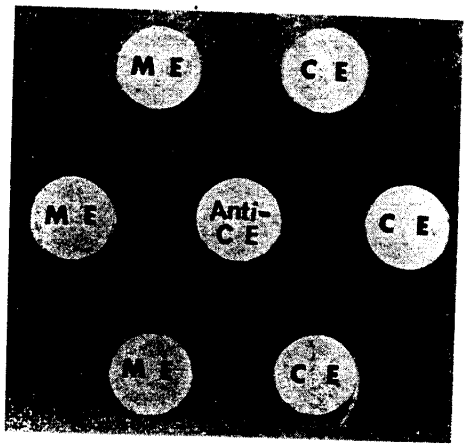


Fig. 4

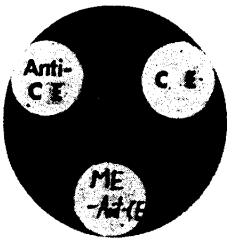


Fig. 5 ME-Ant-CE means the Anti-CE serum absorbed by stomach mucosa sediment (e. g. X 8 indicates eight-fold dilution of antigen)

extracts. Figure 5 illustrates the reactions of the Anti-CE with the CE and with stomach mucosa extract (0 3 mg nitrogen of the antigen) treated with the antiserum (Anti-CE) (0.5 mg nitrogen of the serum). The reactions of the antiserum to these two different extracts reveal several precipitin bands between these two wells. The presence of a band between the Anti-CE and the ME-Ant-CE previously absorbed by the stomach mucosa extract indicates a reactable antigen to be still present in the treated antiserum not being absorbed sufficiently by the excess stomach mucosa antigen. In between the CE well and the ME-Ant-CE well, a weak band is observed, which is continuous with the band between the CE and the Anti-CE, indicating the antigens-antibodies reaction of this immune-system. As can be seen in Fig. 5, the former, weak band is markedly shifted nearer to the ME-Ant-CE well. According to the principle of gel diffusion, a precipitin band appears at the point equivalent to the antigen and the antibody included in the immuneserum. Consequently, the shift of the cancer specific band nearer to the specific cancer precipitin is much less frequently found than nonspecific precipitin antibody, corresponding to the organ or the species specific antigen, if it does exist. From Fig. 5 it may be concluded that the stomach cancer extract has some specific antigen which is not found in normal stomach mucosa extract. Figure 6 shows the results of the attempt made for the elimination of the antibody against normal stomach tissue from the antiserum against stomach cancer. By the absorption with normal stomach extract mixed with the anticancer serum in dilution up to 1 : 16, the cross reaction can be completely eliminated enabling the antiserum to react only with the homologous stomach cancer extract but not with the normal stomach mucosa extract.

Further absorption with the homologous stomach cancer extract caused a complete disappearance of the precipitin bands. These findings are interpreted

that both stomach extract and normal cancer extract contain the same antigenic components though in different quantitative proportions, or the specific cancer antigen is closely related immunologically to the normal stomach cancer antigen. An attempt was also made to demonstrate the characteristics of these antigenic components contained in the stomach cancer extract (Figure 7). The reaction of Anti-CE to the stomach CE and the colon cancer extract (CCE) is represented by two separate bands. One of them, located nearer to antigenic side, seems to indicate a specific immune reaction against the cancer tissue. The other dense band is probably a mixture of multiple antigens-antibodies reactions, some of which are due to the species specific antigen and the organ specific antigen. These findings are common to both CE and CCE.

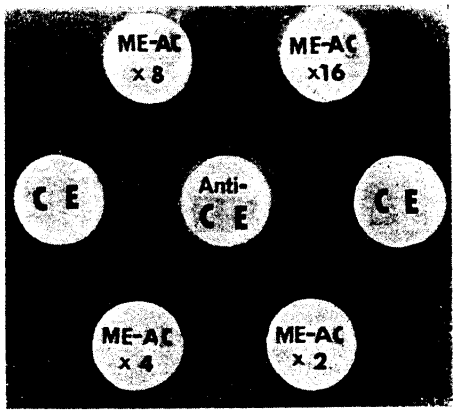


Fig. 6

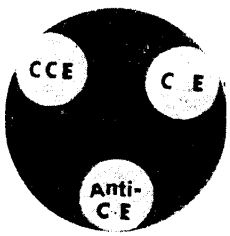


Fig. 7

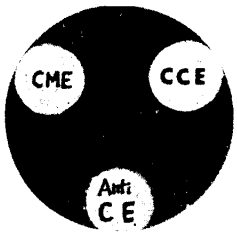


Fig. 8

Figure 8 indicates the difference between the reaction of Anti-CE to CCE and that of Anti-CE to colon mucosa (CME). The illustration shows two different bands; one, located nearer to the antigenic side, is a weak band existing at the CCE side only and not at the CME side, and the other is a moderately complex, dense band, located nearer to the Anti-CE side. The latter band is supposed to be due to such a common antigen present in both colon cancer and colon mucosa as an organ specific (digestive organ mucosa) antigen, species specific antigen and others present in human serum. Therefore, the former probably indicates the cancer specific reaction.

Figure 9 illustrates the reactions of the Anti-CE absorbed by the liver sediment to each cancer of various human organs, such as rectum, breast, thyroid, and colon.

The study indicated that a similar antigen is present commonly in various digestive organs, but absent in non-cancerous tissue or cancer of other organs as well, and it is appropriately called an organ specific antigen of the human digestive organ. An indistinct, faint band exists between the Anti-CE well and the thyroid-cancer-extract (TCE) well, and between the Anti-CE well and the

breast-cancer-extract (BCE) well, apart from the distinct main converging arc of the digestive organ tissue extracts. This band probably indicates the reaction of a cancer specific antibody to the cancer antigen, common to all human cancers.

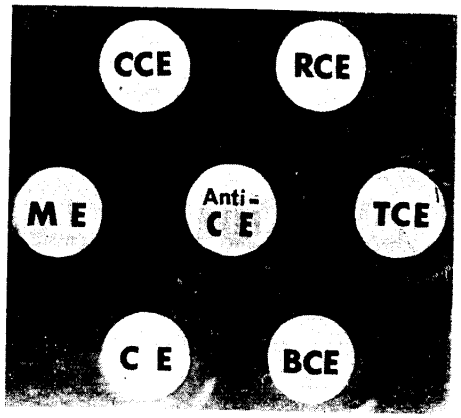


Fig. 9

Figure 10 illustrates the reaction of the treated antiserum (Anti-CE) previously absorbed by human liver sediment with the breast cancer extract and the brain tumor extract. A weak band, previously confirmed to be the cancer specific precipitin band, exists nearer to the breast cancer extract, but not on the side of the brain tumor extract (BTE). The first principle to be derived thus far from these results is that the cancer specific antigenic property may be contained in the stomach cancer extract as one of antigenic components. However, this cancer specific antigen is not present in brain tumor tissue at all, and a little in thyroid and breast cancers. In contrast, in the antigen components there is an organ specific antigen common to various digestive organ mucosas, species specific antigen and others, not identified in the present experiments.

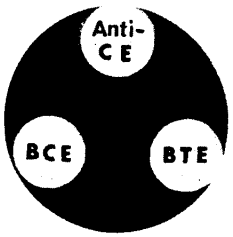


Fig. 10 BTE is the Abbreviation of Brain Tumor Extract

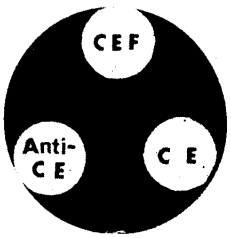


Fig. 11

Figure II presents the result of an attempt made to determine what change occurs in the stomach cancer extract treated by Fluorocarbon from the immunological point of view. Two distinctly different bands such as those observed in the stomach cancer extract are revealed also on the side of the stomach cancer tissue treated with Fluorocarbon, suggesting that the antigen solution is considerably condensed by the Fluorocarbon treatment.

A band indicating non-specific reactions is located nearer to the antiserum side, but the other band is more distinct than that observed with the untreated cancer extract. Figure 12 illustrates the reactions of Anti-CE to the various fractions of the stomach cancer tissue and normal stomach mucosa, isolated by means of differential centrifugation. The reactions are observed as a connected converging arc, surrounding the antiserum (Anti-CE) of the center well. But the arc has a split on the side of thc nucleus fraction, meaning that there exists

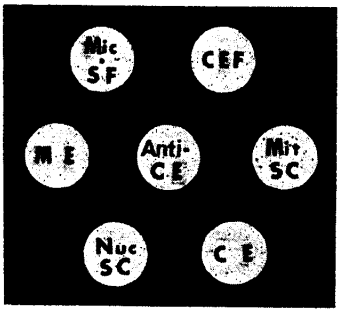


Fig. 12
Mic. SF is the abbreviatitn of microsome fraction and soluble fraction of stomach cancer homogenate. Mit SC is the abbreviation of mitochondria fraction of stomach cancer homogenate. Nuc SC is the abbreviation of the nucleus fraction of stomach cancer homogenate.

nothing reactive to the antiserum. On these plate tests the cancer specific band has not been clearly demonstrated but it might be included in the complex bands of the microsome fraction, the supernatant fraction, and the cancer extract treated with Fluorocardon. Consequently, by this experiment it has been elucidated that the specific cancer antigen is satisfactorily eluted by Fluorocarbon treatment without any loss of the pertinent antigenicity. Moreover, this specific cancer antigen mainly exists in the microsome fraction and the supernatant fraction of cancer tissue. It might then be asked: what chemical characteristics does the specific antigen of human stomach cancer show and its component? Though in the subsequent investigation, it has been demonstrated that the antigen specific to the human cancer is not heat-stable and it is remarkably denatured not only by heating at 56°C for 30 min but also by frequent thawing and freezing as evidenced by the fact that no precipitin band has developed with this antigen solution on the gel diffusion plate. Chemical treatment such as with M/10 formaldehyde solution for 24 hours or with 10% trichloroacetic acid solution for 30 min also seems to alter the nature of the antigenicity.

On the other hand, the antigenic property common to various mucosas of all human digestive organs, the so-called "organ specific antigen", seems to be much more stable and resistant than that of the cancer specific antigen. In the immunoelectrophoresis of the cancer extract treated with Fluorocarbon, the cancer specific antigenic property has migrated to anode, together with other antigenic properties, parallel with the zones of γ-globulin and β_2-globulin. Even by means of DEAE cellulose column chromatography it was impossible to obtain the purified cancer specific antigen.

DISCUSSIONS

In the present study, a pool of 26 specimens served as the source material for the preparation of cancer antigen. Some investigators[17,20] emphasize individual difference in the antigenicity of human cancer tissue so that a single cancer would be more suitable for the inoculation of the rabbit and the same homologous normal tissue for its absorption. This concept seems to be not tenable and

in truth, it[2] might be thought that the tumor specific antigen is concentrated selectively in a pool of specimens, if it exists at all in human cancers commonly beyond negligible individual difference. As an antigen for this study a crude stomach cancer extract, prepared as previously mentioned was used. This preparation does not lose any pertinent antigenicity, namely, it maintains both its soluble and insoluble antigenic properties, because the specimen is prepared not by any elution or extraction but by fractionation. By this method of the inoculation two injections of the crude cancer extract emulsified in Freund's adjuvant[10] coupled with repeated intravenous injections, can well immunize the rabbit to produce fully-satisfactory antibodies in a significantly high titre within 10 weeks in average. When intravenous injections of the antigen solution are further repeated, this significantly-immunized state is usually maintained for the subsequent 2～3 months. The peak period of the immunized state appears between the 10th～14th week, coinciding with the data of TAKEDA[20]. Before bleeding, the resensitization with a large amount of the antigenic solution is done, because the secondary reaction[9,34] can be expected in this case. Freund's adjuvant alone does not produce a favorable cancer specific antiserum against the solid tumor extract like in this experiment, contrary to the data[9,30] with ascites tumor cells, even with the inoculation[15] continued for the period of 6 months, because such a tissue antigen as this may possess weak immunizing properties, making the immunizing period longer than that usually required. Furthermore, cancer specific antigen among the antigen groups contained in the cancer tissue extract seems to be so scarce that the specificity is not well demonstrated by gel diffusion technique. One of the reasons for this weak immunizing property seems to lie in its poor solubility. In an early period of the inoculation, for the first 5 weeks or so, albumin fraction shows no variation, though γ-globulin fraction moderately increases. This phenomenon is somewhat unique as some claim to be[30]. Moreover, it should be noted that the response to the inoculation is of a highly significant value in individual rabbits. In this study (including a preliminary study), nine of eighteen rabbits have not well responded to the inoculation though three rabbits responded remarkably. Recently, the horse is frequently used for the inoculation with such points of advantage as prolonged usefulness and vague immuneserum. There seems to exist some qualitative difference[8] between immunesera of these two animals. As pointed py BLANEY[4] it is one of the most formidable obstacles in this study that such a procedure as immunizing rabbit with heterologous human tissue brings about interspecies or transplantation antibodies.

As HAUSCHKA[18] emphasized in 1952, the combination of precipitation and diffusion in gel is most suitable and useful technique to separate each antigen-antibody reaction in such a mixture of mutiple antigens-antibodies reactions as

that of the stomach cancer tissue and to demonstrate the canccr specific antigen, coupled with differential absorption of the immuneserum. Because of the cancer tissue used here is consisted of a mixture of cancer cells, normal cells, and non-cellular constituents. A principal limitation of the gel diffusion technique is that several bands may migrate with the same speed or dense thick band may mask a weak band nearby so that incomplete resolution results. Moreover, the technique is limited[25,33] to antigen which can diffuse through the gel, that is, being a soluble antigen. To compensate these deficiencies, the antigen treated with aid of ultrasonic disintegrator or by Fluorocarbon was used in the present experiment. The antiserum was carefully purified with absorbing nonspecific precipitins.

On the basis of the result of this experiment, it may be reasonable to conclude that a cancer specific antigenic property exists in the stomach cancer tissues, common to different individuals, different epithelial cancer tissues though the antigenicity may vary in its intensity. This antigenic preperty might be included in the microsome fraction and supernatant fraction and it is heat labile and easliy denatured by chemicals such as formaldehyde solution and trichloracetic acid solution. These observations are interpreted as indicating that the cytoplasmic rather than the nuclear constituents seem to be a likely site for antigenic property characteristic to cancer tissue as illustrated by the studies of TOOLAN[31].

This property might be related to some protein components. Furthermore, this specific property was favorably eluted by means of Fluorocarbon treatment, considerably decreased in nitrogen content and not denatured under this preprration as evidenced by the appearance of a specific precipitin band after this treatment. Therefore, a solution treated in this manner can be used conveniently as the antigen solution for such immunological reactions in order to detect cancer as a "delayed hypersensitivity type of reaction in the skin" and "a reaction of passive anaphylaxis".

ZILBER[37] has recently reported an extensive work on the antigenicity of human stomach cancer by the gel diffusion technique. His results almost agree with the present results, that is, he revealed the cancer specific property, although not absolute, in the alkaline aqueous extracts prepared by centrifugation for 20 min at 4,000 r. p. m. from the ground human cancer tissue. As the nature of this specific property, he did not mention about it on this substance though he performed a few trial experiments but failed to get any conclusion. By means of electrophoresis, he detected an active antigen in the slower fraction that migrated within the zone of γ-globulin and β_2-globulin. From the result of immunoelectrophoresis for the analysis of the antigen solution in the present experiment, the cancer specific property migrated to anode parallel with the zone

γ-globulin. Analytical separation of the various properties was not successful by this method. Nor was it possible to detect a purified cancer specific property from the cancer tissue extract by means of DEAE cellulose column chromatography. In any case, these nonspecific analytical methods are not suitable for the separation of various tissue antigens as this. ZILBFR[36,37] used the agar block method for the purpose of purifying the immuneserum mixed with the homogenized tissue extract for the absorbing procedure. For the purpose of absorbing the nonspecific antibodies, the tissue sediment as mentioned previously is much more favorable and expedient than the tissue extract. In the case with tissue sediment, the treated serum is thoroughly purified by centrifugation at 30,000 r. p. m, for 30 min in the cold room without any fear of denaturation.

On the other hand, BJÖRKLUND[2,3] and his coworker failed to detect the cancer specific antigen in a saline soluble extract of the cancer tissue by means of gel diffusion though they found four cellular antigens in the experiment on human cancer specificity. As a probable reason for their failure in the investigation, it may be that they did not deal with the insoluble substance for the immunization of horse. Namely, the cancer specific property seems to be scarcely soluble in saline, and it is contained in the cancer tissue in a very small amount though it is detectable by the gel diffusion technique. Consequently, it is supposed that the complexity of the antigenic mixture, a very low concentration of the antigen, and the uncertain reactivity of the antiserum reagents pose as the obstacles in analysis of the tissue antigens. At present there are a few observations indicating that human cancer tissue might possess some sort of specificity though no clear-cut results have been obtained so far. These trial experimets were performed by other kinds of reaction such as "complement fixation[17]", "passive anaphylaxis[15]", "delayed type skin reaction[23]" and "cytotoxic effect on cultured cells[3]". BJÖRKLUND[3] and his co-workers, in their early work by means of the gel diffusion as referred above detected four cellular antigens but none of them was specific to cancer. The horse immuneserum, used in their experiments, however, displayed a cytotoxic effect on cancer calls even after the absorption with normal human plasma but it did not show any such effect on normal epithelium from thc same patient. Thereafter, they have carried out extensive experiments on the antigenicity of human malignant tissue with the aid of assay method, cytotoxic effect on cultured cells and reported an evidence of the presence of an antigenic substance connected with insoluble lipoprotein structures on the surface of human malignant cells. BLANEY[4] attempted to reproduce certain portions of BJÖRKLUND's work and was partially successful in it. However, GOLDSTEIN[12] and others, who also tried to reproduce the work of BJÖRKLUND, commented on these data reported by BJÖRKLUND that reactions might be dependent upon the species specific antibody, because he failed to demons-

trate the cancer specific lytic antiserum in the supplementary experiments. Independently, WITEBSKY[35] obtained a lipid antigen by extraction of human stomach cancer with 80% alcohol, which reacted in complement fixation determination with the extracts from cancer tissue and from normal tissue. Even after the absorption with extract from normal tissue, a reaction still occurred with extract of cancer tissue. RAPPORT[26] *et al.* in the studies along the same line, pointed out that lipid antigen contained in the mitochondrial fraction in the case of lymphosarcoma might play an important role in tumor immunology, but from the results of further experiments they suggested that it was an organ and species specific antigen.

Consequently, all these studies ase not sufficiently extensive or precise to permit uncritical acceptance at present. But the results are coincident with the hypothesis that there may be unique cancer antigens even in human cancer and that such antigen may be common to cancer of different individuals. This specificity may be confirmed as being pluralistic one in various immunological reactions.

SUMMARY

From the data presented in this communication, it might be concluded that a cancer specific substance, which can be demonstrated in gel diffusion, is present in human cancer tissue, common to various epithelial cancers of different individuals, although it may vary in its concentration. Needless to say, this substance is quite different from the so-called interspecies antigen or organ specific antigen, as proved by the present experiments. Furthermore, this substance can be eluted well by the Fluorocarbon treatment and it displays physically and chemically unstable characteristics. This substance is likely to be included in the microsome fraction and soluble fraction which was determined by gel diffusion technique. However, the association of this substance with other specific antigenic substances of human cancer, concerned with "delayed type skin reaction", "cytopathogenic antiserum against cancer cell", and "complement fixing antibody in serum of patients with cancer", has not been elucidated in this study.

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