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On the Specificity of Autoantibodies Present in Colon Cancer Patients

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Summary. 223 sera of patients having a colonic cancer or other diseases were examined for the presence of antibodies agglutinating tanned sheep erythrocytes coated with saline extracts of human colonic cancers. Some patients' sera were also investigated for precipitating antibodies. Many positive results were found, mainly but not only, in colonic cancer patients' sera. By absorption studies the antibodies found in these sera have been shown to be directed against an antigen present not only in the tumours but also in normal and foetal tissues as well as in the microsomal fraction of the intestine. The chemical nature of the antigen is still under investigation.

INTRODUCTION

Immunological research on cancer in animals has provided evidence for the occurrence of tumour-specific antigens and antibodies (Korngold and Pressman, 1954; Abelev and Svetkov, 1960; Klein, 1964; Deckers, 1964). Although encouraging in the experimental field, these findings are not paralleled by observations relating to human cancer. The possibility of antibodies to tumours in man has indeed been investigated for over 60 years, and the more recent outstanding contributions include those of Witebsky, Rose and Shulman (1956), Graham and Graham (1955), Zilber (1958), Itoh and Southam (1964), and Perez-Cuadrado, Haberman and Race (1964). Some authors have claimed the detection of antibodies reacting specifically with tumour tissue, while others have reported negative results, e.g. Southam (1965).

Work done in this laboratory has included the study of antibodies to human cancers, both in rabbits immunized experimentally and in the serum of patients with cancer (Bonatti, Rapp and Burtin, 1964; Kleist and Burtin, 1964; Loisillier, Buffe, Tan, Burtin and Grabar, 1966). The present paper is concerned with antibodies in the serum of patients with carcinoma of the colon, reacting with red cells treated with tannic acid and sensitized with extracts of the tumours. We have tried to investigate the significance and specificity of these antibodies, mainly by this method, but also by agar-diffusion precipitation techniques.

MATERIALS AND METHODS

Human sera. Investigations were performed upon sera obtained from the following individuals:

(a) Fifty-three patients with carcinoma of the large intestine. With four exceptions, the tumours were classified histologically as adenocarcinomas. One was a villous papillary

tumour, and the remaining three were squamous carcinomas and may have originated in cells other than those of the mucosa of the large intestine.

(b) Eighty-three patients with carcinoma of various other organs, e.g. stomach, lung, breast, oesophagus, liver and pancreas.

(c) Fifty-three patients with various disorders of the intestines other than cancer, namely ulcerative colitis, chronic diarrhoea, parasitic infestations and benign villous papillomas.

(d) Thirty-four healthy individuals or patients with various non-cancerous conditions.

Human tissues. Apart from a few specimens of normal tissues obtained shortly after death, all the tissues used were removed surgically. Tumour tissue was obtained from eighty-five carcinomas, of which sixty-two were of the large intestine, twenty gastric, two renal and one from an involved part of the liver. In all cases, the carcinomatous nature of the tumour was confirmed histologically. Colonic tissue confirmed histologically as normal was obtained from sixty-five specimens removed for colonic cancer.

Tissues regarded as normal included colons removed for benign tumours or dolichocolon (ten cases), and six colons obtained at necropsy from young children; gastric tissue from four patients undergoing gastrectomy for duodenal ulcer; normal kidneys (three cases) and heart (one case) obtained at necropsy, and a normal spleen. Tissues were obtained also from 3 to 6-month-old foetuses, and included intestine, stomach, liver, kidney, heart and lung.

Extraction of tissues

(a) Buffered-saline extracts were prepared from all tissues. Colonic and gastric mucosa was dissected from the muscular coat and freed as far as possible from submucosal connective tissue before extraction. Several foetal stomachs or intestines were pooled and extracted without previous dissection. Tumours were separated from adjacent normal tissue and as much blood as possible was removed by washing repeatedly in saline containing 10 mg sodium merthiolate per 100 ml.

Tissues were homogenized in 0·1 M phosphate buffer at pH 8·2, in a mechanical 'Ultraturrax' TP 18/2 homogenizer (Janke and Kunkel, K.G., Stauffen i. Br., Germany) at 24,000 rev/min. The homogenates were centrifuged at 40,000 ***g*** for 20 minutes in an M.S.E. 'Speed 18' type centrifuge, and the precipitate was re-homogenized and re-centrifuged. The two resultant extracts and the precipitate were lyophilized.

(b) Phenol extraction was performed by Perlmann and Broberger's (1962) modification of the method described by Westphal, Luderitz and Bister (1952). Homogenates of colonic cancer and of normal colon, prepared as described above, were extracted with an equal volume of 90 per cent phenol for 45 minutes at 65° with mechanical stirring. After centrifugation the watery layer was removed and the phenolic extraction repeated. The watery fractions were lyophilized after precipitation with 3–4 volumes of ethylalcohol containing some grams of sodium acetate (the minimum required to give precipitation).

A microsomal fraction of normal colonic mucosal homogenate was prepared by differential centrifugation in 0·25 M sucrose, the microsomes being deposited at 35,000 ***g*** in a Spinco model L ultracentrifuge. Electron microscopic examination showed the preparation to contain a few other cell constituents, including mitochondria.

Red cell stromata were prepared from pooled red cells including all ABO groups.

Passive haemagglutination techniques

For the detection and titration of antibodies in the patients' sera, we used the passive

haemagglutination technique of Boyden (1951). Sheep red cells (Pasteur Institute) treated with tannic acid were sensitized with lyophilized tissue extracts reconstituted to concentration of 1·5 mg protein per ml (Biuret), according to Kunkel and Ward (1950).

Tests for antibody reacting with phenol extracts of tissues were performed by the haemagglutination technique of Keogh, North and Warburton (1947); this antigen was found to give optimal sensitization of normal (non-tanned) red cells when used at a concentration of 250 μg of dry powder per ml.

For both haemagglutination techniques 0·15 M phosphate buffer at pH 7·4 was used. For washing and suspending the sensitized cells, and for preparing two-fold dilutions of sera (starting at 1 in 10), 1 per cent of normal rabbit serum was added to the phosphate-buffered saline.

Precipitation tests

Precipitating antibodies were detected by the agar diffusion technique of Ouchterlony (1958) and by immunoelectrophoresis in 1·5 per cent agar, using a 0·15 M veronal buffer at pH 8·2 (Grabar and Williams, 1953).

Absorption of sera

Sera were submitted to absorption with the following preparations: (a) Extracts of normal foetal and adult stomach, liver, spleen, kidney, colon and heart, and foetal lung; these extracts were used in various concentrations up to 180 mg protein per ml of serum; (b) extracts of carcinomas, e.g. of the colon, stomach, kidney, at concentrations up to 100 mg protein per ml of serum; (c) lyophilized pooled red cell stromata, 90 mg protein per ml of serum; and (d) killed bacteria (Pasteur Institute), e.g. *E. coli*, *Sp. proteus*, staphylococci, streptococci, all at a concentration of $6 \cdot 1^9$ per ml of serum.

Enzyme treatment of tissue extracts

The saline extracts of tissues were subjected to treatment with trypsin at pH 7·3, pepsin at pH 2·0, and amylase, elastase, DNase, RNase, and papain+cystein, all at pH 7·0. Treatment was for 12–24 hours at 37°, usually with an enzyme concentration of 1 per cent; where necessary, EDTA or DFP was then added to stop enzyme activity. The enzymes were prepared commercially and their purity was not investigated.

RESULTS

HAEMAGGLUTINATION TESTS

The results of titrations upon all the sera, using as antigen red cells sensitized with pooled extracts of colonic carcinomas (TC antigen), are shown in Fig. 1. It is apparent that high titres of antibody occurred more often with sera from patients with colonic carcinoma than with sera from the other groups of patients. Thus 52 per cent of the colonic carcinoma cases had serum titres of 1 in 160 or more, as compared with 17 per cent and 13 per cent for the groups of patients with other cancers and non-cancerous intestinal disorders respectively, and 3 per cent (one of thirty-four) for patients with diverse diseases or healthy individuals.

Five of the seven high titre sera from patients with non-cancerous intestinal disorders were from patients with ulcerative colitis. As Broberger and Perlmann (1959) have described the occurrence in patients with ulcerative colitis of serum antibodies which agglutinate red cells sensitized with phenol–water extracts of colon, comparative haemagglutination tests were performed on sixteen ulcerative colitis sera and thirteen colonic

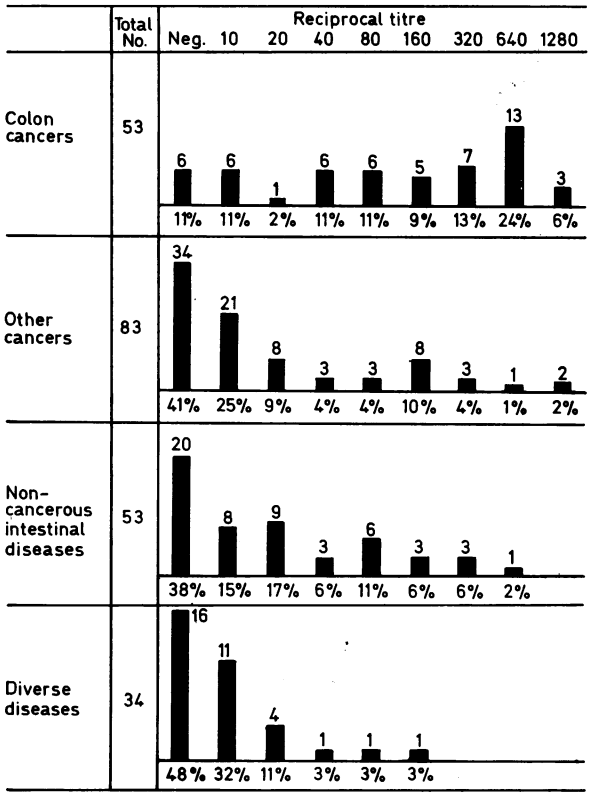


FIG. 1. Titres given by sera in haemagglutination tests with red cells coated with saline extract of colonic cancer (TC antigen).

TABLE 1

TITRES OF ULCERATIVE COLITIS SERA IN PASSIVE HAEMAGGLUTINATION TESTS

No. of serum	Antigen		
	TC	PT	PN
1	640	—	160
2	320	10	160
3	160	80	10
4	160	20	160
5	160	40	80
6	80	20	20
7	40	20	10
8	40	80	40
9	20	20	40
10	20	10	40
11	20	160	160
12	20	10	40
13	10	10±	40
14	0	0	80
15	0	20	160
16	0	20	20

Antigens used to coat red cells: Saline extract of colonic cancers (TC), phenol extract of colonic cancers (PT), and phenol extract of normal colonic mucosa (PN).

TABLE 2
TITRES OF SERA FROM CASES OF COLONIC CANCER IN PASSIVE HAEMAGGLUTINATION TESTS

No. of serum	Antigen		
	TC	PT	PN
1	640	10	20
2	160	20	40
3	320	10	20
4	640	10	40
5	640	0	80
6	20	20	80
7	80±	10	40
8	640	20	40
9	320	80	20
10	40	80	80±
11	80	80	40
12	640	40	40
13	10	40	40

Abbreviations as in Table 1.

cancer sera, using as antigens saline extracts of colonic cancer (TC antigen), and phenol extracts of colonic cancer (PT antigen) and of normal colonic mucosa (PN antigen). The results (Tables 1 and 2) show no obvious relationship between serum titres for the three antigens.

ABSORPTION STUDIES

Sera with titres of 1 in 160 or more in the haemagglutination test with TC antigen were retested following absorption with saline extracts of various normal tissues, foetal tissues, cancers, and with red cell stromata, bacteria, and a microsomal preparation of colonic mucosa. The results are illustrated in Tables 3 and 4.

(a) *Extracts of normal tissues.* Absorption with extracts of normal stomach, kidney, colon, liver or spleen, all in a concentration of 30 mg protein per ml of serum, resulted in falls of titre of only one or two serum dilutions. In a concentration of 120 mg protein per ml of serum, normal colonic extract abolished the haemagglutinating activity of serum, and gastric mucosal extract reduced the titre from 1 in 320 to 1 in 10. By contrast, splenic extract at a concentration of 180 mg protein per ml of serum did not lower the titre significantly.

TABLE 3
EFFECT OF ABSORBING WITH VARIOUS TISSUE EXTRACTS UPON THE TITRES OF SERA FROM THREE PATIENTS WITH COLONIC CANCER

Sera	Titre before absorption	Antigen (minimal concentration)							
		TC 30 mg/ml	CN 120 mg/ml	TE 100 mg/ml	EN 120 mg/ml	TR 120 mg/ml	RN 170 mg/ml	SP 180 mg/ml	LI 180 mg/ml
1	320	0	0	0	10±	0	0	160	10
2	640	0	–	0	0	0	10±	160	80
3	640	0	10±	–	0	0	10±	–	–

Concentrations of antigenic extracts represent the minimal required for complete or almost complete absorption, or the maximal amounts used.

Absorption tests performed with saline extracts of colonic cancer (TC), normal colonic mucosa (CN), gastric cancer (TE), normal gastric mucosa (EN), renal cancer (TR), normal kidney (RN), spleen (SP) and liver (LI).

TABLE 4

TITRES OF SERA FROM PATIENTS WITH COLONIC CANCER AFTER ABSORPTION WITH KILLED BACTERIA

Sera	Titre prior to absorption	Bacteria (6×10^9/ml)				
		Staph.	Strept.	Coli.	Prot.	Prot. + Coli.
1	320	80	160	160	80	160
2	160	160	160	80	80	160
3	320	80	160	80	80	320

Staph. = staphylococci; Strept. = streptococci; Coli. = coliform bacilli; Prot. = proteus bacilli.

(b) *Extracts of tumours.* Pooled saline extracts of colonic tumours, 30 mg protein per ml of serum, completely abolished the haemagglutinating activity. For other cancers, 60–90 mg protein per ml of serum were required to produce this effect. The serum agglutinating titres for red cells coated with phenol extracts of normal tissue or colonic cancer were only slightly lowered by absorption with saline extracts of tumours.

(c) *Foetal tissues.* The saline extracts prepared from the normal foetal tissues, used at a concentration of 120 mg/ml of serum, abolished haemagglutinating activity.

(d) *Microsomal fraction of colonic mucosa.* Haemagglutination was abolished by absorbing the serum with this impure preparation in a concentration of 10–15 mg protein per ml of serum.

(e) *Bacteria.* Absorption with the suspensions of killed bacteria in some instances did not lower the haemagglutinating titre, while in others the titres were reduced by one or two serum dilutions (Table 4).

(f) *Red cell stromata.* Absorption with lyophilized red cell stromata, 90 mg protein per ml of serum, did not reduce the serum titres.

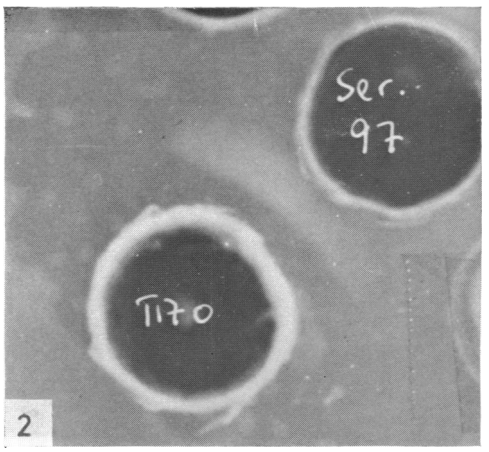
PRECIPITATION TESTS

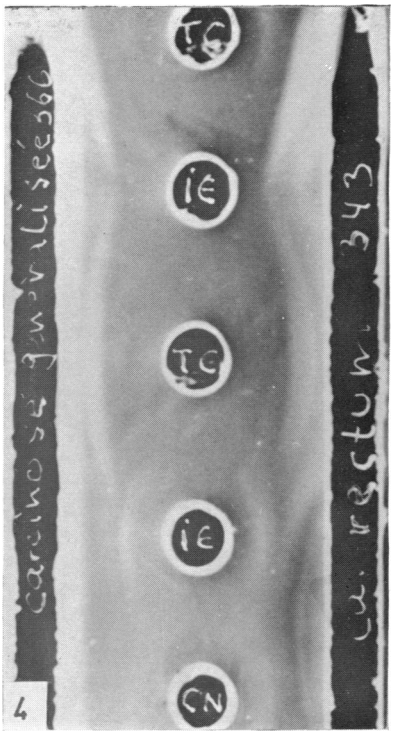
Several patients' sera were investigated for precipitating antibodies reactive with saline extracts of their own tumours or with extracts of other tumours. In three instances we were able to demonstrate strong precipitation lines with the autologous system (Figs. 2 and 3). At least eight sera gave precipitates with the heterologous extracts, but unfortunately not all were strong enough to be demonstrated in the photographs (Fig. 4).

TABLE 5

EFFECT OF TREATING ANTIGENIC SALINE EXTRACTS OF COLONIC CANCER WITH VARIOUS ENZYMES

Enzyme	Effect on haemagglutination titre
Trypsin	None
Pepsin	None
Amylase 10%	Partial
Amylase 1%	None
Elastase	None
Papain + cystein	Partial
DNase	Partial
RNase	Partial





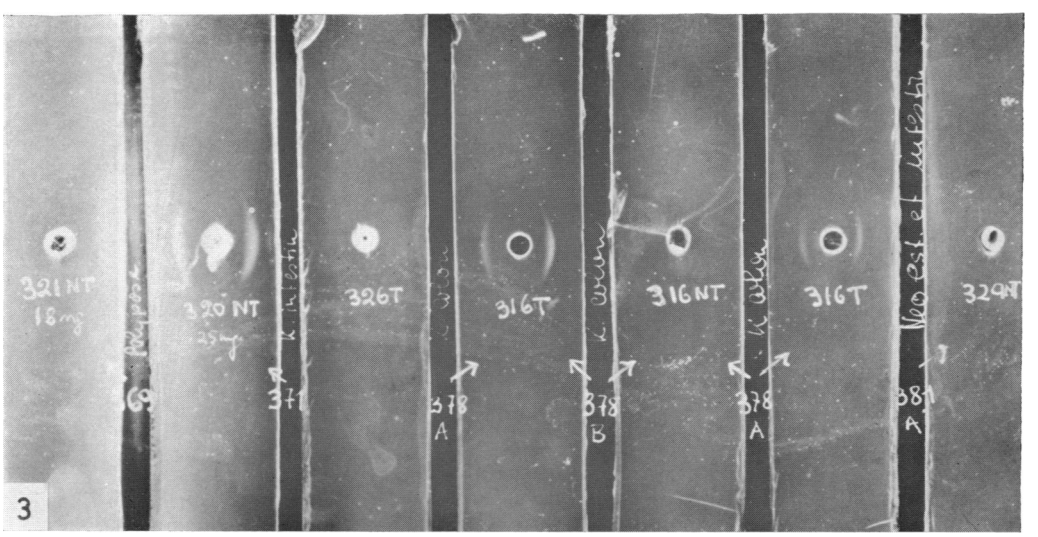


FIG. 2. Agar double diffusion reaction between the serum and the tumour of the same patient.

FIG. 3. Immunoelectrophoresis of colonic cancer extracts. Patients' sera in troughs. Autologous sera and tumour extracts are designated by arrows.

FIG. 4. Agar double diffusion study. The reservoirs contain, from top to bottom, extracts of colonic cancer (TC), foetal intestine (IE), gastric cancer (TE), and normal colonic mucosa (CN). The left trough contains serum from a patient with generalized carcinomatosis, and the right trough contains serum from a patient with cancer of the large intestine.

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CHEMICAL STUDY OF THE ANTIGEN

Results of preliminary attempts to characterize the antigen by enzymic studies are shown in Table 5. Positive sera were titrated following absorption with the enzyme-treated preparations of TC antigen. A fall in haemagglutinating titre of two or more dilutions was considered to indicate at least partial resistance of the antigen to the particular enzyme.

DISCUSSION

The results of passive haemagglutination tests upon serum from patients with colonic cancer indicate the occurrence of antibody reactive with an antigen present not only in saline extracts of colonic and other cancers, but also in normal adult and foetal tissues, and in a microsomal fraction of colonic mucosa. Differences in the removal of the antibody by absorption with extracts of various normal tissues and tumours appear to be of a quantitative nature, and the antigen is present in higher concentration in extracts of colonic cancers than in extracts of normal colonic mucosa and some other normal tissues (Table 3).

The incidence of high haemagglutinating titres (1 in 160 or more) was much greater (52 per cent) in sera of patients with colonic cancer than in control sera (3 per cent) (Fig. 1), and this suggests an immune response by the host against the tumour. The results of absorption tests with red cell stromata and bacteria do not support the alternative possibility that blood group isoantibodies or bacterial antibodies are concerned.

If circulating antibodies have resulted from an immune response to the tumour, then it is necessary to explain why sera from 11 per cent (six out of fifty-three) of cases of colonic cancer reacted weakly, and 11 per cent gave negative results. Factors possibly concerned relate to the state of the tumour, its histological type, and the treatment of the patient. As regards the state of the tumour, Graham and Graham (1955) have observed the demonstration of antibodies to be extremely difficult in individuals with extensive or necrotic cancers or where there was a general decrease in the patient's resistance. If antibodies are produced by such patients, they might be fixed on to the tumour or blocked by circulating antigens. On the other hand, a well-localized tumour might release little or no antigen and might thus not stimulate antibody formation.

Of our twelve sera yielding low titre or negative results, one was from a patient with a degenerated villous carcinoma, two were well-localized tumours, and two were rather necrotic, yet we did observe high titres in other cases with a necrotic tumour.

Two sera giving a negative result were from patients with a squamous cancer, but the third patient with squamous cancer had a serum titre of 1 in 160.

As regards treatment, it is well known that carcinostatic chemotherapeutic agents, are, like X-rays, cytotoxic to leucocytes, and depress the formation of antibodies. Some authors, for example Kondo and Ischihashi (1964), have demonstrated the harmful effects of certain of these agents in man and have shown that their administration to animals can result in abolition of defence mechanisms. Some of our patients received chemotherapy, but in others there was no history of such treatment to account for negative results. Nor have we found any evidence of an increase of autoantibody titres following radiotherapy, as reported by Finney, Beyers and Wilson (1960).

Fourteen sera giving high titres of haemagglutination were from patients with cancer in sites other than the colon. The two with highest titres had respectively secondary

cancer in the liver, and secondary cancer involving the oesophagus and originating in the breast. In the others, cancer originated in the digestive tract (five cases), the pancreas (one case), the liver (three cases) and epidermoid cancer of the lung (three cases). A close embryonic relationship between organs of the digestive tract might provide a link between most of these cases, but would not explain the high titres in the three cases of lung cancer.

Of particular interest to us were the high titres of haemagglutination in the group of patients with ulcerative colitis (Table 1). Evidence of an increased incidence of colonic cancer in this condition has been provided since 1929. The reported incidence of cancer varies from 0·6 to 14·2 per cent (Goldgraber, Humphreys, Kirsner and Palmer, 1958), while in a recent report the incidence was put at 19 per cent (Goldgraber and Kirsner, 1964). Because of the similarity of symptoms of the two conditions, colonic cancer supervening on ulcerative colitis is liable to remain undiscovered for a long time. It therefore seems possible that the high serum titres observed in some of our cases of ulcerative colitis might indicate progress towards malignancy. It is of interest that Perez-Cuadrado, Habermann and Race (1965) reported antibodies produced in rabbits and reacting specifically with human colonic tumours; the antibodies reacted also with colonic tissue from a patient with ulcerative colitis, and the authors have interpreted this as indicating pre-cancerous change.

The comparative studies with saline and phenol extracts of normal colon and colonic cancer, carried out upon sera from cases of ulcerative colitis and of colonic cancer, show that at least two different antigen–antibody systems are probably involved, since the titres of sera for saline and phenol extracts appeared unrelated (Tables 1 and 2). Moreover, absorption by saline extracts left almost untouched the haemagglutinating activity for cells coated with phenol extracts. Unfortunately, we were unable to remove completely, by absorption with large quantities of phenol extracts, haemagglutinating activity for cells coated with phenol extracts, a possible explanation being denaturation of the antigen during phenol extraction.

In view of the 3 per cent of high titre reactions with sera from patients without colonic cancer, and the low titres and negative results for some colonic cancer patients, the haemagglutination test seems of little diagnostic value. A low serum titre does not exclude colonic cancer, nor does a high titre confirm its presence. The few results obtained in precipitation tests suggest the occurrence of precipitating autoantibodies reacting with colonic cancer, but their specificity has not been investigated and their significance is unknown. It must be emphasized that in one instance a serum reacted to give a precipitate with an extract of foetal intestine, and some other sera reacted with non-cancerous or normal colonic extracts. We must therefore conclude that we are not able to demonstrate, by the two methods used, antigen or antibody specific for colonic cancer. Nevertheless, antibodies have been demonstrated and their development might be attributable to release of cellular constituents, normally confined within cells, as a result of invasion of tissues by the malignant tumour, entry of such released constituents into the lymphatic or blood streams thereby stimulating an antibody response. This would explain the reactivity and absorbability of the antibodies with normal adult and foetal tissues.

It is too early to draw, from the results of the preliminary enzyme studies, any firm conclusions on the chemical nature of the antigen.

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