DEMONSTRATION OF TUMOR-SPECIFIC ANTIGENS IN HUMAN COLONIC CARCINOMATA BY IMMUNOLOGICAL TOLERANCE AND ABSORPTION TECHNIQUES*

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PLATES 35 TO 39

Numerous attempts have been made by previous workers to demonstrate the presence of tumor-specific antibodies in sera obtained from animals immunized with preparations of human cancers (1–8). Such demonstrations, if consistently reproducible, would indicate the existence in human cancer tissue of unique homologous antigens not present in normal tissue, and might thus lead to a better understanding of the nature of the neoplastic process.

One of the techniques frequently employed in this type of investigation is the absorption of antitumor antiserum with normal tissue in order to remove antinormal components of the antiserum (1, 9, 10). Any residual antibody activity in the absorbed antiserum which is directed against tumor material is then considered to be tumor-specific. However, in interpreting the results of absorption experiments, little consideration has been given to 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.

Another, more recent, technique for the demonstration of tumor-specific antibodies is the utilization of animals rendered immunologically tolerant to normal tissues during neonatal life (11). These tolerant animals are then immunized with tumor preparations of the same donor species. In those cases where adequate suppression of the immune response to normal tissue components has been achieved, immunization of the tolerant animals with tumor preparations during adult life has led to the development of antibodies apparently specific for the tumor (12–21). Nevertheless, the reported studies of human cancer employing acquired immunological tolerance (17–21) have left certain points unclear. In particular, the greatest cause for possible misinterpretation of results has been the failure to use the same donors as the source of normal and tumor material. Instead, the normal tissue has come from non-cancerous individuals

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or from cadavers free of malignant neoplastic disease. Despite the fact that normal individual-specific antigens may have been diluted out in a tissue pool, it is conceivable that some of the antibodies labeled "tumor-specific" in these experiments were, in reality, the result of individual antigenic differences between donors.

The objectives of the present study were (a) to attempt to demonstrate the presence of tumor-specific antigens in carcinomata of the human colon utilizing both the absorption and immunological tolerance techniques with normal and tumor tissues obtained from the same human donors; and (b) to compare the sensitivity and reliability of each of these two methods for the immunological demonstration of unique tumor constituents. Neither of these two objectives has been previously attempted.

Materials and Methods

The Preparation of Normal and Tumor Tissue Extracts.—The source of tissue in this series of experiments was human colon. The specimens removed surgically for primary adenocarcinoma of the colon, were taken from various sites ranging from the cecum to the rectosigmoid junction. The diagnosis was confirmed postoperatively by histological examination in all cases. Carcinoma of the colon was chosen for study because it almost never extends submucosally more than 6 to 7 cm on either side of the tumor visible in the gross (22). Thus, portions of the specimen more than 7 cm proximal or distal to the cancerous growth could be considered normal tissue. In this way the problem of individual-specific antigenic differences between the normal and tumor tissue pools was overcome. Random microscopic examination of the areas of tissue considered to be normal failed to reveal the presence of cancer cells at any time. The 7 cm portion of tissue normal in the gross on either side of the neoplasm was removed and discarded. Only the central, obviously cancerous portion was used as tumor tissue for extraction. Normal tissue extracts were prepared from sections of colon more than 7 cm distant from the visible edges of the tumor.

Two pools of tissue, each consisting of ten specimens, were collected and extracted by somewhat different methods. The extraction procedure followed for each pool, however, was identical for the normal and tumor tissues except that the two aliquots were stored and handled apart from one another in order to avoid contamination. Donors of all major blood groups were represented in each pool. In addition to the two pools of tissue, twelve individual specimens which had not been included in either pool were extracted by the procedure used for pool I tissues.

Extraction Procedure for Pool I.—The mucosal lining of each specimen was stripped as cleanly as possible from the underlying tissue. In order to make the normal and tumor tissue extracts as similar as possible, small amounts of muscularis mucosae and stroma were included in the normal tissue strips because the tumor material invariably contained these structural components. The tissue strips were cut into small pieces with stainless steel scissors and washed clean in cold physiological saline solution. The finely cut pieces were then put through a hand-operated stainless steel tissue grinder fitted with a fine sieve which held back a large proportion of the connective tissue elements. The resultant mince was suspended in 0.03 M phosphate-saline buffer at pH 8.0 in proportions of 1 gm wet weight of mince to 4 ml of buffer, and then homogenized in 120 ml aliquots in a Virtis "45" homogenizer at 45,000 RPM for 15 minutes at 4°C. The homogenate was centrifuged at 1000 g for 1 hour at 4°C, the supernatant decanted and then stored in sterile bottles. The sediment was resuspended in the phosphate-saline buffer (2 ml per gm of the initial wet weight of the mince), thoroughly stirred and then exposed in 10

ml aliquots to ultrasonic vibration at 38.5 kc per second for 3 minutes in a fixed frequency Sonogen ultrasonic transducer. The ultrasonated suspension was again centrifuged as outlined above and the supernatant added to that obtained after the initial centrifugation. The pooled supernatants made up the tissue extracts of pool I. Bacteriological cultures were performed and both the normal and tumor tissue extracts showed very light growths of *Escherichia Coli.* After the addition of streptomycin in quantities of 15 μg/ml, repeated cultures of the extracts revealed no bacterial growth. Kjeldahl determinations were performed on the extracts to determine protein content. Finally, the pools were divided into 10 ml aliquots, quick frozen in a dry ice-alcohol bath and stored at $-20°C$ as stock material for the procedures outlined below.

Extraction Procedure for Pool II.—The procedure followed for pool II was identical with that of pool I until the stage where the supernatants of the two centrifugation steps were combined. From this stage the procedure differed as follows:

The pooled supernatants were again subjected to centrifugation, but this time in a Beckman model "L" preparative ultracentrifuge at 22,000 g (Rav) for 32 minutes (precipitation time). The supernatant was decanted and lyophilized. Prior to use the powdered material was reconstituted with distilled water and filtered through 0.22 μ Millipore filters (type HA) into sterile bottles. Bacteriological cultures of the filtrates were invariably sterile. Kjeldahl determinations were performed and the protein concentrations adjusted according to needs prior to use.

Immunization of Adult Rabbits.—Twenty adult, male, New Zealand white rabbits weighing 2.0 $\pm$ 0.2 kg were divided into 5 groups of 4 each. The different groups were immunized with the following materials: normal tissue extract of pool I (N_1), tumor tissue extract of pool I (T_1), normal tissue extract of pool II (N_2), tumor tissue extract of pool II (T_2), and pooled human plasma (P). (The pooled human plasma was obtained from 30 normal donors representing all major blood groups.)

The injections, 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 complete Freund's adjuvant. 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.

Induction of Immunological Tolerance to Normal Human Colon Extract in Neonatal Rabbits.— Six mature, New Zealand white female rabbits were purchased at 2 weeks of pregnancy. These animals were kept isolated and were cared for by the same individual at all times. Within 29 $\pm$ 1 days of fertilization 40 young rabbits were born to the 6 does. Of these, 24 (60 per cent) survived until the completion of the experiment. Within 12 hours of birth, 19 of the neonatal animals that eventually survived were given subcutaneous injections of normal tissue extract of pool I (containing 10 mg of protein) together with 12 mg of pooled human plasma and 3 mg of human hemoglobin. These injections were repeated every 12 hours for a total of 4 doses. The injections of human blood components were given in order to increase the possibility of induction of tolerance to (*a*) blood components contaminating the tissue extracts, and (*b*) tissue components containing antigenic determinants similar to those of plasma proteins. Further subcutaneous injections of normal tissue extract alone (containing 5 mg of protein) were given at 3, 7, 10, 14, 21, 28, 55, and 60 days of age. These animals were bled at 72 days of age to determine whether an immunological response had been obtained against normal tissue. Five of the neonatal animals which served as controls received no injections during this period of the experiment.

Immunization of the Apparently Tolerant Rabbits.—At 80, 84, 87, 90, and 140 days of age, 14 of the 19 survivors of the experimental group received 1 ml injections of tumor tissue extract of pool I (containing 5 mg of protein) emulsified in an equal volume of complete Freund's adjuvant. Injections of 0.1 to 0.2 ml were given into a foot-pad and the remainder intramus-

cularly into a flank. Five animals of the experimental group and the 5 controls received similar injections of normal tissue extract containing 5 mg of protein. All the animals were bled from their marginal ear veins at 100 and 150 days of age and the sera stored for testing.

Techniques of Antibody Demonstration.—The Ouchterlony technique (23) of double diffusion in agar gel was performed in 1 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 on one occasion only. The antigen concentration used was 10 mg protein per ml in all cases unless otherwise specified. Initial incubation of the plates was carried out in a moist environment at 37°C for 24 hours to encourage diffusion of materials from the wells. Patterns were then allowed to develop in the same humid atmosphere at 25°C for 7 days.

Immunoelectrophoresis (23) was performed in perspex molds filled to a depth of 2 mm with 1 per cent agar gel in either 0.1 M tris buffer at pH 8.6 or in 0.1 M acetic acid-acetate buffer at pH 4.0. The usual pattern consisting of a central trough with a well on either side was cut in the gel. The wells were filled with 0.05 ml of the test antigens and subjected to electrophoresis at 6 to 7 v per cm for 90 minutes in the same buffer system used to prepare the agar gel. The plate was then removed from the electrophoresis apparatus and the trough filled with test antiserum. The precipitin patterns were allowed to develop in a moist environment at 25°C for 7 days.

Hemagglutination reactions were carried out by a modification of the technique described by Sehon (24) in which antigen is covalently coupled to rabbit erythrocytes with bis-diazotized benzidine (BDB). In all tests performed, the erythrocytes were sensitized by mixing aliquots of either the normal or tumor tissue extracts (containing 1.5 mg of protein dissolved in 3 ml of saline) with 0.1 ml of a 50 per cent suspension of washed rabbit red cells and 0.5 ml of BDB-phosphate buffer solution at pH 7.3. In certain tests the technique was slightly modified by adding the BDB to the erythrocytes immediately before rather than after the addition of the antigen.

Passive cutaneous anaphylaxis (PCA) reactions were performed in mice by a modification of the procedure of Ovary (25) as described by Halpern *et al.* (26). Intracutaneous injections of 0.02 ml of test serum were followed in 3 hours by intravenous injections of a mixture containing 0.3 ml of 0.7 per cent solution of Evan's blue (T1824) and 1 mg of the test antigen protein. Reactions were read 30 minutes after the intravenous injection by sacrificing the animal and measuring the largest diameter of the subcutaneous dye spot. The following grading system was used: ±, less than 4 mm; +, 4 to 8 mm; ++, 8 to 12 mm; +++, 12 to 16 mm; and ++++, greater than 16 mm. If a positive reaction was observed the titer was obtained by determining the highest dilution of antiserum which gave a positive (+) reaction.

In the immunofluorescent studies, antiserum-fluorescein conjugation was performed as described by Rinderknecht (27) and non-specific staining material removed by the technique of Goldstein *et al.* (28). The conjugates were used only if they fulfilled the criteria of specificity outlined by Kaplan (29). Small blocks of tissue to be stained and examined were snap-frozen at −40°C and frozen sections, 6 to 8 μ in thickness, were cut on a Lipshaw electric cryostat and microtome model 1500. Sections were thawed on microscopic slides at room temperature and fixed in ether-alcohol (equal parts) for 10 minutes at room temperature followed by incubation in 95 per cent alcohol for 30 minutes at 37°C. The sections were then dried in air and stored for 2 to 24 hours at 4°C until used. The indirect or "sandwich technique" of Coons (30) was carried out using sheep anti-rabbit γ-globulin antiserum[1] conjugated to fluorescein (27). The sections to be studied were first covered with a few drops of unconjugated test antiserum and incubated

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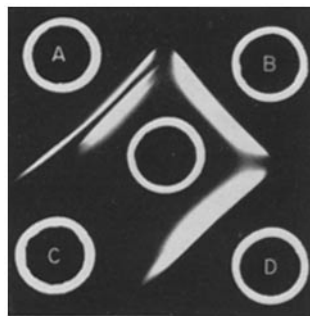[1] The sheep anti-rabbit γ-globulin antiserum was kindly provided by Dr. L. Gyenes of our laboratory.

in a moist environment at 37°C for 20 minutes. The sections were then washed in changes of buffered saline at pH 7.3 for 20 minutes at which time the staining and washing procedure was repeated with the fluorescein-conjugated sheep anti-rabbit γ-globulin antiserum. The sections were dried in room air and inspected by ultraviolet fluorescence microscopy using a Leitz dialux fluorescence microscope with a high vacuum Osram burner HBO 200 as the illuminating system. Ultraviolet filters 4 mm UG1 and/or 2 mm UG2 were used in conjunction with a protective filter system. Ocular magnification 10 was used permanently with various objectives (magnification expressed as ocular X objective). Photomicrographs were taken with a Leica M2 camera and adapter using Kodak plus X black and white film.

Absorption and Dilution of Antiserum.—In all the absorption reactions performed, the absorbing antigen was in solution rather than in the dry state. In order to obtain uniformity of results all antisera, whether absorbed or not, were diluted to four times their original volume before the performance of any test. In the case of the hemagglutination reactions the dilutions noted are those of whole antiserum prior to the addition of any absorbing medium. In those cases where a heavy precipitate formed during the absorption procedure, the sample was centrifuged at 2000 RPM for 15 minutes and the clear supernatant used in the various tests.
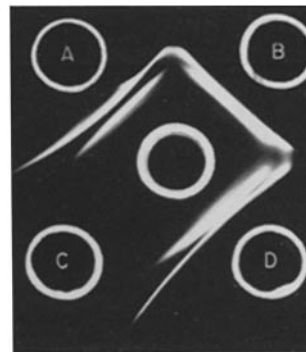
Preliminary Characterization of Antigens and Antibodies.—The method of Winzler et al. (31) for extraction of mucoprotein with 0.6 M perchloric acid was applied to the antigen preparation of pool I (T_1). The filtrate prepared by this method was dialyzed against buffered saline at pH 7.3 for 24 hours. The antitumor antisera prepared by absorption and by immunological tolerance were each fractionated by column chromatography on sephadex G-200 (32) and were each treated with mercaptoethanol according to the technique of Deutsch and Morton (33).

RESULTS

Experiments Employing Non-Tolerant Rabbits and the Absorption Technique.—
Text-figs. 1 and 2 show the Ouchterlony patterns developed between the anti-

TEXT-FIG. 1   TEXT-FIG. 2

TEXT-FIG. 1. Center, N_1, (A) n_1, (B) t_1, (C) control serum from non-immunized rabbits, (D) p. A marked degree of cross-reactivity is shown by the three antisera against the normal antigenic mixture.

TEXT-FIG. 2. Center, T_1, (A) n_1, (B) t_1, (C) control serum from non-immunized rabbits, (D) p. A marked degree of cross-reactivity is shown by the three antisera against the tumor antigenic mixture.

gens of pool I (N_1, normal tissue extract of pool I, T_1, tumor tissue extract of pool I, and P, pooled human plasma) and their corresponding antisera (n_1, t_1, and p respectively) (Table I). As expected, a multiplicity of precipitin bands appeared in each antigen-antibody system. It is apparent from examining these patterns that a high degree of antigenic cross-reactivity exists between the normal and tumor tissue extracts and their respective antisera. It will also be noted that both the normal and tumor extracts contained many components which produced precipitin lines with anti–human plasma antiserum. Absorption of each of the three antisera (n_1, t_1, and p) was then carried out with an excess of both N_1 (2 ml containing 10 mg of protein) and P (1 ml of whole human plasma) so that complete inhibition of precipitin band formation was achieved

TABLE I

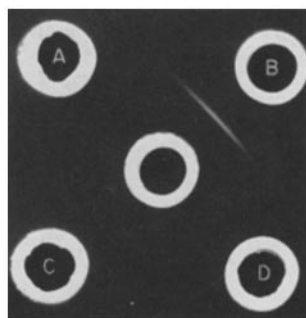*Summary of Symbols used in the Text and for Figure Captions*

Symbols	Explanation of symbols
N_1, N_2	Extracts of normal colonic tissue of pool I and pool II respectively.
T_1, T_2	Extracts of colonic tumor tissue of pool I and pool II respectively.
P	Pooled human plasma.
n_1, n_2	Rabbit antisera against N_1 and N_2 respectively.
t_1, t_2	Rabbit antisera against T_1 and T_2 respectively.
p	Rabbit antiserum against P.
trs-150	Pooled antisera of rabbits rendered almost completely tolerant to N_1 and then immunized with T_1 (at 150 days of age).
-abs	This suffix following the symbol for an antiserum indicates that it has been absorbed with an excess of normal colonic tissue extract and pooled human plasma.

against the absorbing antigens. The anti–tumor tissue antiserum (t_1) so treated (and denoted henceforth as t_1-abs), gave a single band against T_1. However the absorbed n_1 (n_1-abs) and absorbed p (p-abs) failed to do so (Text-fig. 3). In order to determine whether the bacterial flora of the bowel or the streptomycin used to sterilize the extract were playing a role in the production of the apparently tumor-specific band, further absorption of t_1-abs was carried out with an ultrasonated extract of pooled bowel flora to which streptomycin had been added. Such treatment failed to alter the single band between T_1 and t_1-abs. Similarly, absorption of t_1-abs with human fibrinogen failed to alter the band formation. However, the addition of T_1 (0.5 ml containing 5 mg of protein) to t_1-abs prior to performing the Ouchterlony reaction, specifically inhibited the appearance of the precipitin band between T_1 and t_1-abs.
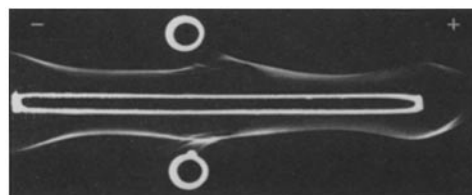
The immunoelectrophoretic patterns obtained between the tissue extracts N_1 and T_1 and the anti–tumor tissue antiserum (t_1) before and after inhibition with both N_1 and P are shown in Text-figs. 4 and 5 respectively (electrophoresis

carried out in tris buffer at pH 8.6). As with the Ouchterlony patterns numerous bands appeared against both antigenic mixtures prior to absorption, but only a single band remained between T_1 and t_1-abs after the absorption procedure. The antigen taking part in this precipitation showed little or no mobility at the buffer pH of 8.6. However, when the immunoelectrophoretic studies between T_1 and t_1-abs were repeated with acetic acid-acetate buffer at pH 4.0, the single band was found nearer the negative pole.

The results of the hemagglutination reactions are listed in Table II. It will be noted that both n_1 and t_1 showed very high titers against their homologous antigens as well as a high degree of cross-reactivity between normal and tumor

TEXT-FIG. 3. Center, T_1, (A) n_1-abs, (B) t_1-abs, (C) absorbed control serum from non-immunized animals, (D) p-abs. The only remaining band is seen between T_1 and t_1-abs.

TEXT-FIG. 4. Immunoelectrophoresis in agar gel (tris buffer at pH 8.6). Upper well, N_1, lower well, T_1, trough, t_1. A multiplicity of bands is observed between the antiserum and both antigenic mixtures.

TEXT-FIG. 5. Immunoelectrophoresis in agar gel (tris buffer at pH 8.6). Upper well, N_1, lower well, T_1, trough, t_1-abs. A single residual band is seen between T_1 and t_1-abs.

tissues. Following the absorption procedures, especially, the titers obtained were markedly dependent upon the manner in which the erythrocyte sensitization was performed. If T_1 was coupled to the rabbit red cells by mixing the antigen with the erythrocytes prior to the addition of BDB, negative results were obtained with t_1-abs. However, if the red cells and BDB were mixed immediately before the addition of the antigen T_1, a relatively high titer was obtained between T_1 and t_1-abs. Regardless of the method of sensitization of the erythrocytes, negative results were obtained between N_1 and n_1-abs or t_1-abs; and between T_1 and t_1-abs further absorbed with T_1.

TABLE II

Immunological Reactions with Sera of Non-Tolerant Animals

Antigen	Anti-serum	Absorbing antigens	Hemagglutination		PCA	
			Titer A*	Titer B‡	Skin reaction	Titer§
N_1	n_1	—	1.64×10^6	13.10×10^6	++++	128
T_1	n_1	—	1.64×10^6	6.55×10^6	++++	128
N_1	t_1	—	0.82×10^6	6.55×10^6	++++	128
T_1	t_1	—	1.64×10^6	13.10×10^6	++++	128
N_1	n_1	$N_1 + P$	0	0	0	—
T_1	n_1	$N_1 + P$	0	0	0	—
N_1	t_1	$N_1 + P$	0	0	0	—
T_1	t_1	$N_1 + P$	0	0.20×10^6	++	16
T_1	t_1	$N_1 + P + T_1$	0	0	0	—

* Hemagglutination titer observed when erythrocytes were sensitized by the addition of BDB *after* mixing red cells with antigen.

‡ Hemagglutination titer observed when erythrocytes were sensitized by the addition of BDB *before* mixing red cells with antigen.
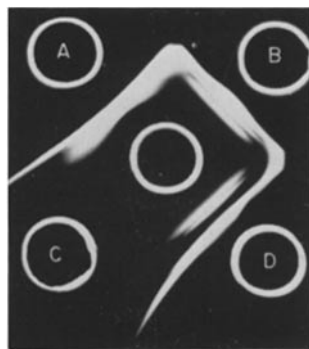
§ Reciprocal of the highest dilution of antiserum giving a positive (+) PCA reaction.

The results of passive cutaneous anaphylaxis (PCA) experiments (Table II) correlated well with those obtained by agar gel diffusion and hemagglutination. High degrees of reactivity and cross-reactivity were observed between normal and tumor tissue extracts and their respective antisera. Following the absorption of the antisera with N_1 plus P, the only remaining positive reaction was that of t_1-abs challenged with T_1. This reaction could be inhibited specifically by absorbing t_1-abs with T_1 prior to injection.

Comparison of Results Obtained in Non-Tolerant Rabbits with Antigens of Pool I, Pool II, and Twelve Individual Specimens of Adenocarcinoma of the Colon.—The Ouchterlony patterns obtained with pool II antigens (N_2 and T_2) and their homologous antisera (n_2 and t_2 respectively) are shown in Text-figs. 6 and 7. The patterns are similar to those observed with pool I materials (Text-figs. 1 and 2). Following absorption of each of the three antisera (n_2, t_2, and p)
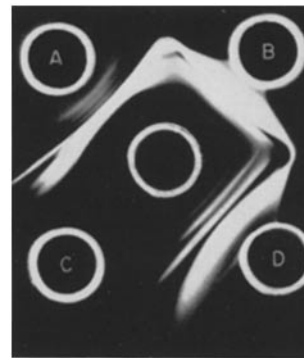
with an excess of both N_2 (2 ml containing 10 mg of protein) and P (1 ml of whole human plasma) the only antigen-antibody combination which was found to produce precipitation in agar gel was T_2 against t_2-abs (Text-fig. 8). It will be noted that a single precipitation band was obtained. In order to determine whether or not the same antigen-antibody combinations were responsible for the tumor-specific bands obtained in the agar gel studies of both pool I and pool II, Ouchterlony reactions were carried out using T_1 and T_2 against t_1-abs (Text-fig. 9) and T_1 and T_2 against t_2-abs (Text-fig. 10). In both cases reactions of identity were obtained.

TEXT-FIG. 6

TEXT-FIG. 7

TEXT-FIG. 6. Center, N_2, (A) n_2, (B) t_2, (C) control serum from non-immunized rabbits, (D) p. A marked degree of cross-reactivity is shown by the three antisera against the normal antigenic mixture.
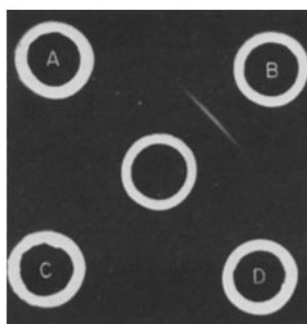
TEXT-FIG. 7. Center, T_2, (A) n_2, (B) t_2, (C) control serum from non-immunized rabbits, (D) p. A marked degree of cross-reactivity is shown by the three antisera against the tumor antigenic mixture.

Further adsorption of t_1-abs and t_2-abs on their corresponding lyophylized normal tissue extracts (100 mg of extract protein/ml of antiserum) prior to testing failed to change the patterns illustrated in Text-figs. 9 and 10. Similarly, increasing the protein concentrations of both the normal and tumor tissue extracts from 10 mg protein/ml to 40 mg protein/ml during the reaction did not alter the appearance of the band formation.
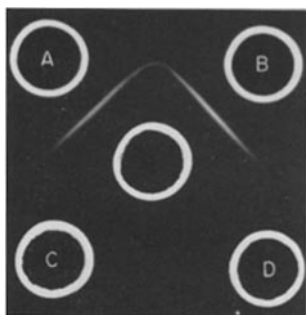
Immunoelectrophoretic studies of N_2 and T_2 against t_2-abs (in tris buffer at pH 8.6) showed the development of a single band between T_2 and t_2-abs (Text-fig. 11). The reactive antigen like that of T_1 against t_1-abs showed little or no mobility at pH 8.6 (compare with Text-fig. 5).

When the normal tissue extracts of the twelve individual specimens were subjected to double diffusion in agar gel against t_1-abs and t_2-abs, no precipitin band formation was observed. However, when the tumor extracts of these
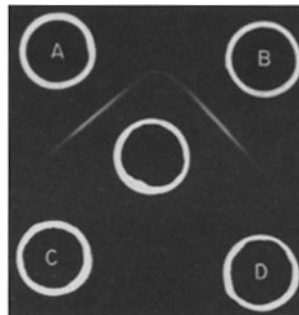
specimens were tested against either t_1-abs or t_2-abs, it was found that each of the twelve tumor extracts formed single bands against both antiserum preparations. The twelve precipitin bands thus formed showed reactions of identity between themselves and with the pooled tumor extract preparations T_1 and T_2

Text-Fig. 8. Center, T_2, (A) n_2-abs, (B) t_2-abs, (C) absorbed control serum from non-immunized rabbits, (D) p-abs. The only remaining band is seen between T_2 and t_2-abs.
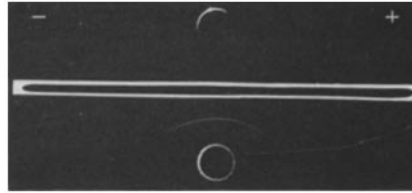

Text-Fig. 9   Text-Fig. 10

Text-Fig. 9. Center, t_1-abs, (A) T_1, (B) T_2, (C) N_1, (D) N_2. The bands formed between t_1-abs and each of the two tumor antigenic mixtures show a reaction of complete identity.

Text-Fig. 10. Center, t_2-abs, (A) T_1, (B) T_2, (C) N_1, (D) N_2. The bands formed between t_2-abs and each of the two tumor antigenic mixtures show a reaction of complete identity.
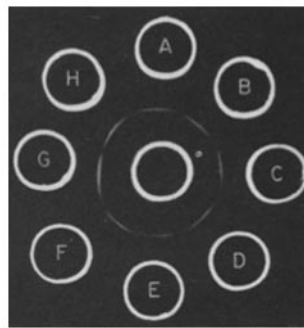
(Text-fig. 12). The results obtained against t_1-abs were identical to those obtained against t_2-abs. Furthermore, when the tumor extract of any of the twelve individual specimens was used to further absorb t_1-abs or t_2-abs, there was specific inhibition of band formation against T_1 and T_2.

Experiments with Rabbits Rendered Tolerant to Normal Tissue.—Sera obtained at 72 days of age from the animals receiving injections of N_1 from birth produced no precipitin bands when reacted against N_1 in agar gel. These tests were repeated with sera obtained at 100 days of age. (The experimental group

received its initial immunizing injections of T_1 at 80 to 90 days). At this time each of the 14 animals of the experimental group showed a single band against N_1 and three bands against T_1 (Text-fig. 13). A form of split and/or partial tolerance (34, 35) to normal tissue had thus been induced in these animals. The first control group which had received N_1 injections from birth and had been given the same material between 80 and 90 days of age also showed a

TEXT-FIG. 11. Immunoelectrophoresis in agar gel (tris buffer at pH 8.6). Upper well, N_2, lower well, T_2, trough, t_2-abs. A single band is seen between T_2 and t_2-abs.
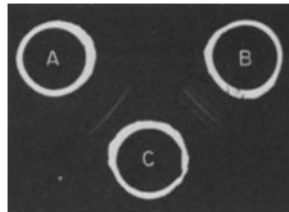

TEXT-FIG. 12. Center, t_2-abs, (A) T_1, (B) T_2, (C-H) tumor extracts of six of the twelve individual specimens of human colonic adenocarcinoma. Reactions of complete identity are seen between all the precipitin bands which formed. Identical reactions were obtained with the other six specimens.

single band against N_1. The non-tolerant control group which received its initial injections of N_1 between 80 to 90 days of age showed a number of bands against N_1 at 100 days (Text-fig. 14).
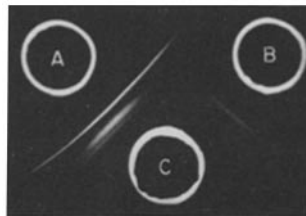
At 150 days, the serum of each of the animals of the experimental group was found to form two precipitin bands against N_1 and six bands against T_1 (Text-fig. 15). Split and/or partial tolerance had thus been maintained toward the normal tissue extract. Because of the similarity of the patterns obtained with each of the individual animal sera, the sera were pooled for the remainder of the tests performed and will be referred to henceforth as trs-150. Absorption of trs-150 with human fibrinogen and an ultrasonated extract of bowel flora containing streptomycin failed to alter the pattern described above. The Ouchter-

lony patterns produced by sera from the two control groups were similar to those seen at 100 days except that sera from the animals of the group that received only N_1 from birth now produced two rather than one band against N_1 (Text-fig. 16).

The results of the hemagglutination reactions of the 150 day bleeding are shown in Table III. Once again much greater sensitivity was obtained when

TEXT-FIG. 13. (A) N_1, (B) T_1, (C) Pooled antisera of rabbits showing split and/or partial tolerance to N_1 at 100 days of age. Only a single band developed against the normal antigenic mixture whereas three bands were formed against the tumor extract.
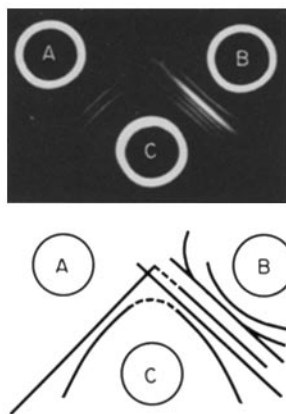

TEXT-FIG. 14. (A) Pooled antisera of control group (100 days of age) which received initial injections of N_1 between 80 to 90 days of age, (B) Pooled antisera of control group (100 days of age) injected with N_1 from birth, (C) N_1.
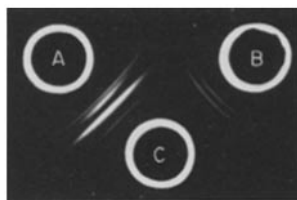
the rabbit erythrocytes were sensitized by mixing the BDB with the red cells immediately before the addition of antigen. Much higher titers were observed when trs-150 was reacted with red cells sensitized with T_1 than those sensitized with N_1.

The PCA reactions (Table III) again correlated well with the results of double diffusion in agar and hemagglutination experiments. Sites sensitized with trs-150 gave a $++++$ reaction when challenged with T_1 as compared to a $\pm$ reaction when challenged with N_1.

Comparison of Results Employing the Absorption Technique with Those Utilizing Tolerance.—The precipitin pattern obtained by the absorption technique is compared to that developed by the tolerance method in Text-fig. 17. The single tumor-specific band between T_1 and t_1-abs showed a reaction of identity with two of the bands formed between T_1 and trs-150. Similar reactions

TEXT-FIG. 15. (A) N_1, (B) T_1, (C) trs-150. Accompanying drawing shows reactions of identity not well shown in the photograph. Two bands developed against the normal antigenic mixture, but six bands developed against the tumor extract.

TEXT-FIG. 16. (A) Pooled antisera of control group (150 days of age) which received initial injection of N_1 between 80 to 90 days of age. (B) Pooled sera of control group (150 days of age) injected with N_1 from birth. (C) N_1.

TABLE III

Immunological Reactions with Sera of Tolerant Animals (trs-150)

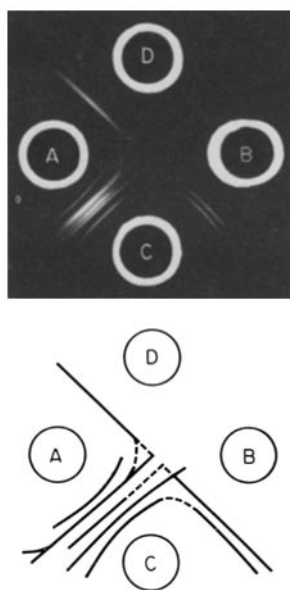| Antigen	Antiserum	Absorbing antigen	Hemagglutination		PCA	
			Titer A*	Titer B‡	Skin reaction	Titer§
N_1	trs-150	—	3200	25,600	±	—
T_1	trs-150	—	6400	1.64×10^6	++++	64
N_1	trs-150	N_1	0	0	0	—
T_1	trs-150	N_1	0	0.20×10^6	++	16
T_1	trs-150	T_1	0	0	0	—

* Hemagglutination titer observed when erythrocytes were sensitized by the addition of BDB *after* mixing red cells with antigen.

‡ Hemagglutination titer observed when erythrocytes were sensitized by the addition of BDB *before* mixing red cells with antigen.
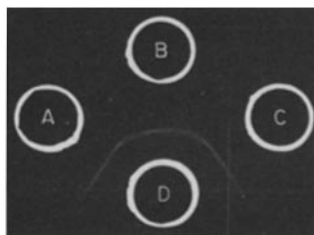
§ Reciprocal of the highest dilution of antiserum giving a positive (+) PCA reaction.

of identity were present for the two bands formed between N_1 and trs-150 and for two of the six bands between T_1 and trs-150. Therefore, two of the six precipitin lines produced by T_1 and trs-150 have no counterparts in the zones between the other wells.

When trs-150 was absorbed with an excess of N_1 (1 ml containing 5 mg of protein) and reacted in agar gel with T_1 (Text-fig. 18), two bands remained. Both of these bands gave a reaction of identity with the tumor-specific bands
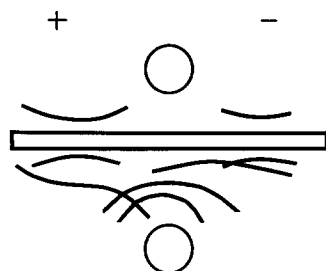

Text-Fig. 17. Comparison of results obtained by the tolerance and absorption technique (A) T_1, (B) N_1, (C) trs-150 (D) t_1-abs. Accompanying drawing shows reactions of identity not well shown in the photograph. It can be seen that two of the six bands between T_1 and trs-150 have no counterparts in the areas between the other reactant wells.
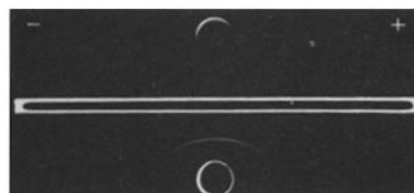

Text-Fig. 18. (A) t_1-abs, (B) trs-150 absorbed with N_1, (C) t_2-abs, (D) T_1. The two bands formed between T_1 and trs-150 absorbed with N_1 show reactions of identity with the apparently single bands formed between T_1 and both t_1-abs and t_2-abs.

obtained between T_1 and t_1-abs and between T_1 and t_2-abs. A similar demonstration of this phenomenon can be seen from Text-figs. 19 and 20 which show the immunoelectrophoretic patterns formed between the antigens of N_1 and T_1 and trs-150 before and after absorption with N_1 (tris buffer at pH 8.6). The lone residual band of Text-fig. 20 corresponds closely to those seen in Text-figs. 5 and 11 prepared from absorbed non-tolerant rabbit antisera. It should be noted that one of the two bands observed on agar gel diffusion between T_1

TEXT-FIG. 19. Immunoelectrophoresis in agar gel (tris buffer at pH 8.6). Upper well, N_1, lower well, T_1, trough, trs-150. Line drawing is shown because in a number of the bands formed were not intense enough to be faithfully reproduced by photography.
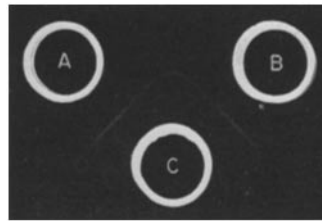

TEX-FIG. 20. Immunoelectrophoresis in agar gel (tris buffer at pH 8.6). Upper well, N_1, lower well, T_1, trough, trs-150 absorbed with N_1. A single residual band developed between T_1 and trs-150 absorbed with N_1.

and trs-150 absorbed with N_1 (Text-fig. 18) could not be demonstrated by immunoelectrophoresis.

Results of Immunofluorescent Studies.—Histological sections through adjacent normal and tumor areas of the twelve specimens of adenocarcinoma of the colon, which had not been included in either of the two tissue pools, were studied by the indirect or "sandwich" technique. The test antisera used in each case included n_1, n_2, t_1, t_2, the various absorbed preparations of these sera, and trs-150. The results obtained with any given test antiserum preparation was almost identical for each of the pathological specimens. Furthermore no significant differences could be detected between the immunological properties of antisera prepared against pool I antigens or against pool II antigens.

Figs. 1 to 9 are representative of such findings. The specimen was removed from a patient with adenocarcinoma of the transverse colon. The use of either unabsorbed n_1 or unabsorbed t_1 as test antisera resulted in intense staining of both tumor tissue and normal colonic tissue (Figs. 1 to 4). When t_1-abs was employed as the test antiserum little or no fluorescence could be found in the normal tissue areas (Fig. 5), whereas the tumor areas still showed strong fluorescein labeling (Fig. 6). The degree of fluorescence of the tumor tissue was somewhat less intense than before absorption. After absorption of t_1-abs with T_1, little or no residual fluorescence could be found in the tumor tissue (Fig. 7).

Reactions carried out with trs-150 showed that only a slight degree of fluorescence was imparted to the normal tissue areas (Fig. 8), whereas the tumor tissue was intensely labeled (Fig. 9). A comparison of Fig. 6 with Fig. 9 would suggest that the serum of the experimental group of animals showing

TEXT-FIG. 21. (A) T_1, (B) filtrate of T_1 treated with 0.6 M perchloric acid, (C) t_1-abs. A reaction of complete identity can be seen between the two bands formed.

split and/or partial tolerance to N_1 caused stronger labeling than the absorbed tumor-specific antiserum originally obtained from non-tolerant animals.

Preliminary Characterization of Tumor-Specific Antigens and Antibodies.— Following treatment of T_1 with 0.6 M perchloric acid, the dialyzed filtrate, after concentration to the original volume of T_1 employed, produced a single band in agar gel against t_1-abs. This band showed a reaction of complete identity with the band formed between T_1 and t_1-abs (Text-fig. 21).

Fractionation of t_1-abs and trs-150 on a sephadex G-200 column yielded the usual 3 peaks (32). The entire antibody activity of each antiserum preparation, as determined by agar gel diffusion against T_1, was found to be in the second (7S) peak. The Ouchterlony patterns were identical to those observed with whole t_1-abs and whole trs-150 against T_1. The first (19S) and third (3-4S) peaks were totally inactive. Upon treating t_1-abs and trs-150 with 0.1 M mercaptoethanol, Ouchterlony and hemagglutination reactions against T_1 were found to give the same results as had been obtained prior to treatment.

DISCUSSION

The experimental findings would suggest that adenocarcinomata of the human colon contain unique tumor antigens which are absent from normal

colonic tissue, and are capable of stimulating tumor-specific antibody formation in heterologous animals.

Tumor-specific antibodies were demonstrated by the techniques of agar gel diffusion, immunoelectrophoresis, passive cutaneous anaphylaxis, and hemagglutination in antitumor antisera which had been absorbed with normal colonic tissue components and pooled human plasma. Other investigators using either the absorption technique (9, 10, 36) or other methods (8, 37–41) have obtained results essentially similar to those reported here. However, in a critical evaluation of these and related studies, Southam (42) has pointed out that the results must be interpreted with caution "because an adequate normal control tissue is seldom obtainable." This source of error was overcome in the present investigation because both normal and tumor tissues were obtained from the same donors. It was also shown in this study that the tumor-specific antibodies demonstrated by the absorption technique were not directed against bacterial contaminants or against the unusually high concentrations of fibrin found in many neoplastic tissues (43–46). Moreover, the further adsorption of t_1-abs and t_2-abs on large quantities of lyophylized normal tissue extract (100 mg of extract protein/ml antiserum) failed to inhibit the development of the tumor-specific band formation between these antisera and the pooled tumor extracts (T_1 and T_2). These observations strongly suggest that the tumor-specific antigens under consideration are qualitatively tumor-specific and not merely present in higher concentrations in tumor tissue than in normal tissue.

Comparison of the apparently single tumor-specific precipitin bands observed in agar gel between pool I antigens (T_1) and t_1-abs and between pool II antigens (T_2) and t_2-abs indicated that the same antigen-antibody complexes were responsible for the production of both bands. This finding would suggest the existence of one or more common tumor-specific antigens in the pool I and pool II tumor aliquots. Furthermore, the immunofluorescent studies provided additional evidence for the presence of the same common antigen(s) in twelve individual tumor sections which had not been included in either of the two tissue pools. The qualitative similarity of the tumor-specific antigens in the two tissue pools and the twelve individual specimens was demonstrated by the Ouchterlony technique. It may thus be inferred that when normal colonic tissue undergoes tumor transformation to adenocarcinoma new antigenic moieties develop which are identical for each individual tumor.

It is not possible to conclude from these studies whether the specific tumor antigen(s) found in adenocarcinoma of the human colon are restricted to this neoplasm or are present in other human malignancies. The findings of other workers are of interest in this respect. Korosteleva (9) using antitumor antiserum absorption and complement fixation techniques found that common tumor-specific antigens could be demonstrated in various types of malignant tumors obtained from different individuals. There was, however, no antigen common to all tumors. In another study, Toolan (47) showed that rat antiserum

prepared against several human cancer cell lines cross-reacted with the HEp3 cell line. Further evidence to support the concept that different tumor types may contain common antigens is obtained from tissue culture cytotoxicity studies by Bjorklund (48), and from studies of cancer cell homotransplantation in man by Southam and his associates (49, 50). The possibility that the tumor-specific antigen(s) demonstrated in the present investigation may occur in human neoplasms other than adenocarcinoma of the colon is presently under active investigation by the authors.

The experimental results obtained with the sera of rabbits immunized with colonic tumor tissue after split and/or partial tolerance had been induced to normal colonic antigens provided additional evidence for the presence of tumor-specific antigens. The development of incomplete rather than complete tolerance may be attributable to a number of factors. The relatively wide species' difference between rabbit and man is probably the most important single reason for this phenomenon (35). Furthermore, since the normal tissue used to induce tolerance contained numerous antigenic components, it is conceivable that some of these were present in concentrations too low to produce the tolerant state (19, 51). Finally, it should be noted that the first immune response to normal tissue occurred at 100 days. At this point in the immunization schedule, the animals had already received four injections of tumor extract (T_1) emulsified in complete Freund's adjuvant. Paraf (52) has postulated that Freund's adjuvant in addition to its well established action of stimulating antibody production, may be instrumental in breaking immunological tolerance or paralysis in previously suppressed animals. The suggested mechanism is the increased proliferation of antibody producing cells beyond the availability of antigen required to suppress the newly formed cells.

Despite the failure to induce complete immunological tolerance to the normal tissue extract (N_1), the degree of suppression of the immune response was sufficient to demonstrate the presence of specific tumor antigens in the tumor tissue extract (T_1). For example, when the pooled serum of the immunologically suppressed animals was tested by agar gel diffusion, passive cutaneous anaphylaxis, or hemagglutination, more intense reactions were noted against T_1 than against N_1.

Comparison of the tumor-specific antibodies found by the use of the tolerance technique with those found by the absorption technique is of considerable interest. Agar gel diffusion studies revealed six bands between T_1 and trs-150 (Text-fig. 17). Of these six bands, two showed reactions of identity with the two bands between N_1 and trs-150, and thus represented antigenic components common to normal and tumor tissue. Another two of the six bands between T_1 and trs-150 showed reactions of identity with the apparently single tumor-specific band between T_1 and t_1-abs suggesting that the latter band, although apparently single, consisted of at least two antigen-antibody complexes. However, immunoelectrophoretic study of T_1 against t_1-abs yielded only a

single band (Text-fig. 5). It is possible that the failure to demonstrate a second band by immunoelectrophoresis may have been due to a relatively small absolute quantity of one or more of the tumor antigens in the volume of tumor extract used in this procedure. An alternative explanation is that the tumor-specific precipitin reaction between T_1 and t_1-abs involved only a single antigenic molecule containing two or more tumor-specific antigenic determinants. Thus far, four of the six precipitin bands have been accounted for, leaving two apparently tumor-specific precipitin bands between T_1 and trs-150 which were not observed with the absorbed antisera. This demonstration of additional tumor-specific antibody activity in trs-150 as compared with t_1-abs was further confirmed by immunofluorescent, passive cutaneous anaphylaxis, and hemagglutination experiments. Since the same antigenic material, T_1, was used to immunize both groups of animals, the presence of additional tumor-specific antibody activity in trs-150 must be adequately explained.

One possible explanation is that the non-tolerant animals immunized with T_1 failed to produce antibodies against certain of the tumor-specific antigens. Such an occurrence might be explained by the phenomenon of competition between antigens or "crowding out" (53). It has been shown in a number of experimental situations that exposure of animals to multiple antigens, either simultaneously or in succession, may lead to the failure of some of the antigenic components to stimulate antibody production. In the present study, when the non-tolerant animals were injected with tumor tissue extract, the tumor-specific antigens had to compete with the normal tissue components of the tumor in order to stimulate antibody production. Thus, some of the tumor-specific antigens may have been "crowded out." However, in the group of immunologically suppressed animals, the competition faced by the tumor-specific antigens was greatly reduced because antibody production to the normal tissue components of the tumor extract had been markedly suppressed. The second possible explanation for the greater tumor-specific antibody activity of trs-150 as compared to t_1-abs is that although the non-tolerant animals produced antibodies to all the antigenic components unique to the tumor material, some of these may have been removed during the absorption procedure with normal tissue extract and pooled human plasma. The latter hypothesis is probably the more important, since absorption of trs-150 with normal tissue extract reduced its tumor-specific antibody activity to levels comparable to that of t_1-abs.

Therefore it may be concluded that, under the conditions of the present experiment, more tumor antigens were demonstrated by the tissue tolerance technique than by the absorption method. Whether these additional antigens are qualitatively different from normal tissue antigens (54), or merely present in tumor tissue in higher concentrations than in normal tissue has not as yet been determined.

The results of sephadex G-200 fractionation of t_1-abs and trs-150 indicate

that the tumor-specific antibodies have sedimentation coefficients of about 7S. This conclusion is supported by the failure to reduce tumor-specific antibody activity following treatment with 0.1 M mercaptoethanol. Extraction of T_1 with 0.6 M perchloric acid and agar gel diffusion study of the filtrate revealed that at least part of the tumor-specific antigenic preparation was mucoprotein in nature. Previous investigations of other tumor systems (55) have also suggested abnormalities of tumor tissue mucoprotein components.

Because it was found that one or more of the tumor-specific antigenic determinants might be polysaccharide in nature, and since the conventional BDB hemagglutination technique (24) failed to correlate with results of the other serological tests, this procedure was slightly modified (56). During the process of erythrocyte sensitization, the BDB was mixed with the rabbit cells immediately before, rather than after the addition of antigen. The reasons for this change become apparent, if it is remembered that the BDB hemagglutination technique was originally developed for use with protein antigens (56) which are coupled to erythrocytes through stable covalent linkages with their histidine and tyrosine groups. However, it is well known that antigens containing carbohydrates (e.g. bacterial polysaccharides or tannic acid) may coat red cells directly and non-specifically (57, 58). It was felt that if a number of important determinants of the tumor-specific antigens were polysaccharide in nature, then the addition of BDB *after* the antigenic material had been mixed with the erythrocytes would allow some, or all, of the immunologically active groupings to be non-specifically adsorbed onto the red cells. The determinant groups would then be unavailable for union with antibody. However, if the BDB were added to the red cells immediately *before* the addition of antigen, competition would occur between various portions of the antigen molecules to determine which would link to the red cell. The histidine and tyrosine of the protein portion of the molecule would tend to conjugate to the red cell through the BDB linkage, while the non-protein antigenic determinants would be left free to take part in the hemagglutination reaction. The results obtained (compare titers A and B in Tables II and III) would indicate that the proposed mechanism did indeed predominate. It would therefore seem probable that the tumor-specific antigens, although containing protein components probably have polysaccharide determinants of great importance.

SUMMARY

Two methods were used to demonstrate the presence of tumor-specific antigens in adenocarcinomata of the human colon: (a) rabbits were immunized with extracts of pooled colonic carcinomata, and the antitumor antisera thus produced were absorbed with a pooled extract of normal human colon and with human blood components; (b) newborn rabbits were made immunologically tolerant to normal colonic tissue at birth, and were then immunized with pooled

tumor material in adult life. Normal and tumor tissues were obtained from the same human donors in order to avoid misinterpretation of results due to individual-specific antigenic differences.

The antisera prepared by both methods were tested against normal and tumor antigens by the techniques of agar gel diffusion, immunoelectrophoresis, hemagglutination, PCA, and immunofluorescence. Distinct antibody activity directed against at least two qualitatively tumor-specific antigens, or antigenic determinants, was detected in the antisera prepared by both methods and at least two additional tumor antigens were detected exclusively in antisera prepared by the tolerance technique. Whether these additional antigens were qualitatively different from normal tissue antigens, or merely present in tumor tissue in higher concentrations than in normal tissue has not as yet been determined. Furthermore, it was shown that the tumor-specific antibodies were not directed against bacterial contaminants or against the unusually high concentrations of fibrin found in many neoplastic tissues.

It was concluded from these results that the pooled tumor extracts contained tumor-specific antigens not present in normal colonic tissue. Identical tumor-specific antigens were also demonstrated in a number of individual colonic carcinomata obtained from different human donors.

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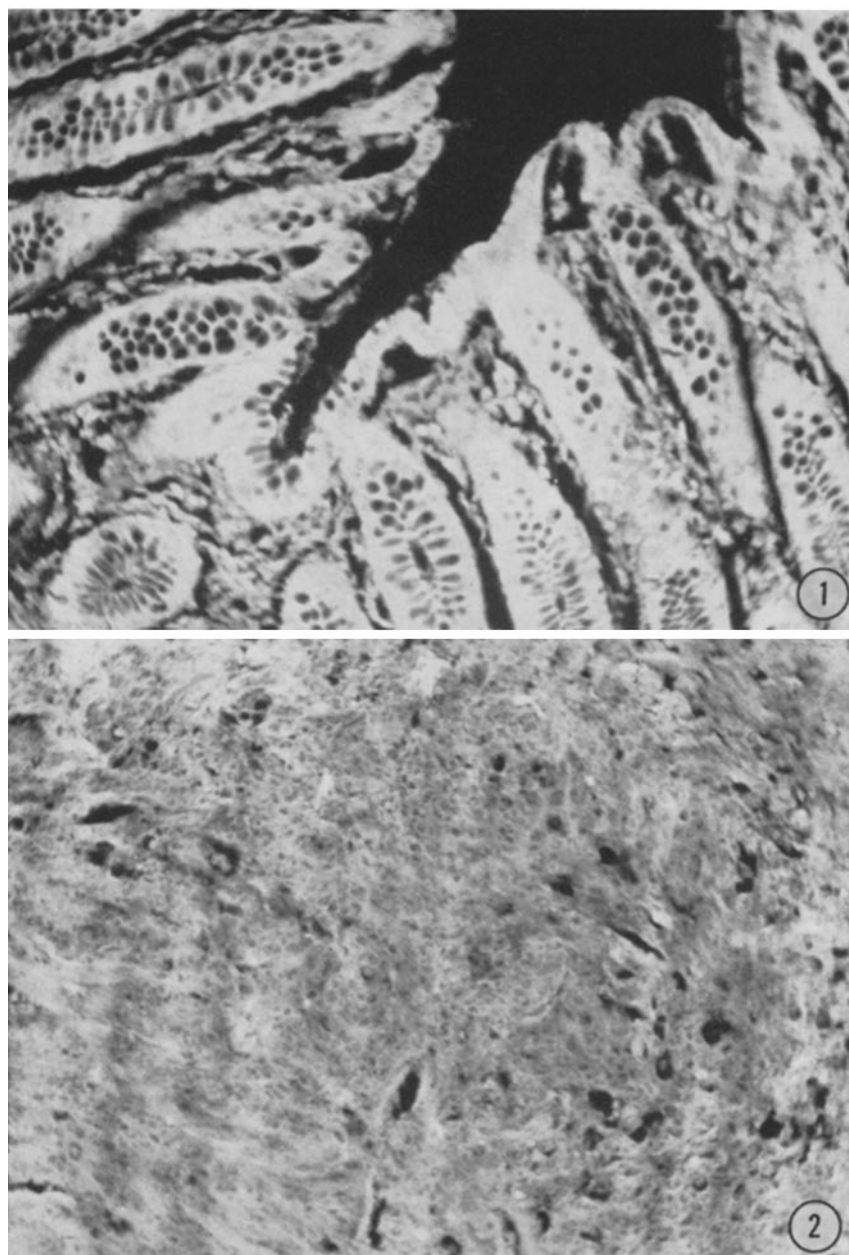
EXPLANATION OF PLATES

PLATE 35

FIG. 1. Section of normal area of colon treated with n_1. Intense fluorescence can be seen throughout the tissue. × 100.

FIG. 2. Section of tumor area of colon treated with n_1. The intensity of fluorescence seen indicates a marked degree of cross-reactivity between some normal tissue and tumor tissue antigens. (Compare staining intensity with that of Fig. 5 and Fig. 7). × 100.
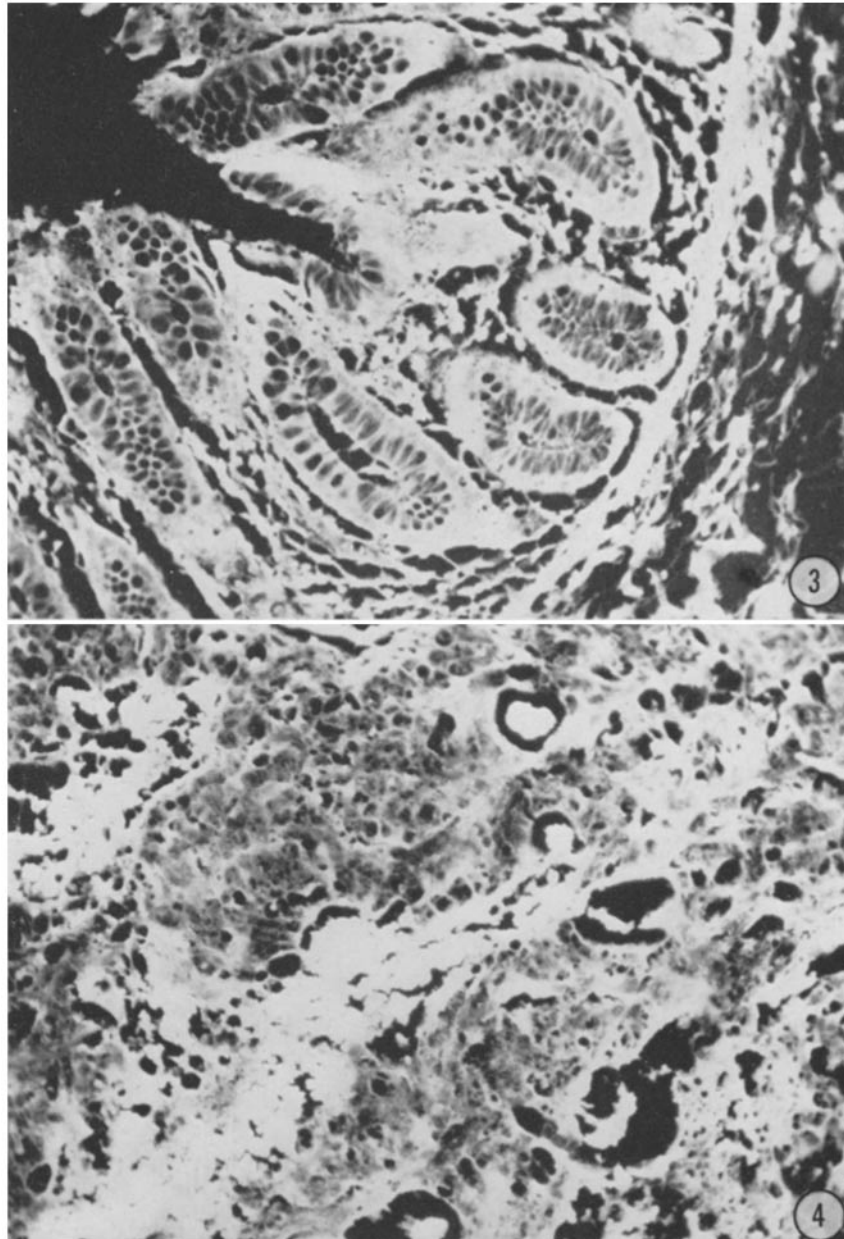
(Gold and Freedman: Tumor-specific antigens)

PLATE 36

FIG. 3. Section of normal area of colon treated with t_1. The degree of the fluorescein label taken up by the normal tissue suggests marked cross-reactivity between some normal tissue and tumor tissue antigens. $\times$ 100.

FIG. 4. Section of tumor area of colon treated with t_1. Diffuse fluorescence is seen throughout the tumor area. $\times$ 100.
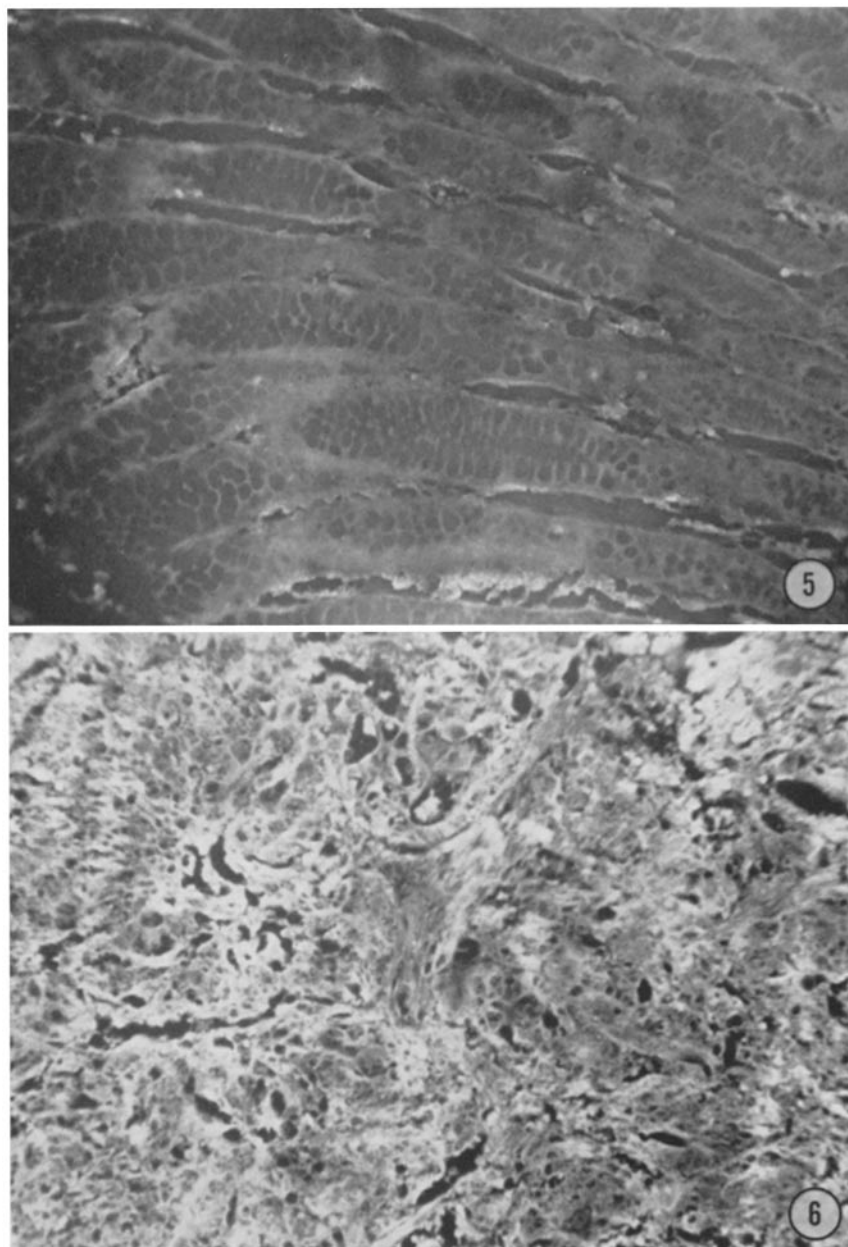
(Gold and Freedman: Tumor-specific antigens)

PLATE 37

FIG. 5. Section of normal colon treated with t_1-abs. Little fluorescein labeling remains, indicating that t_1-abs contains no demonstrable antibodies against normal colonic components. $\times$ 100.

FIG. 6. Section through colonic tumor area treated with t_1-abs. The degree of fluorescent intensity is less marked than in Fig. 4 but remains very distinct, revealing the presence of tumor-specific components in t_1-abs. $\times$ 100.
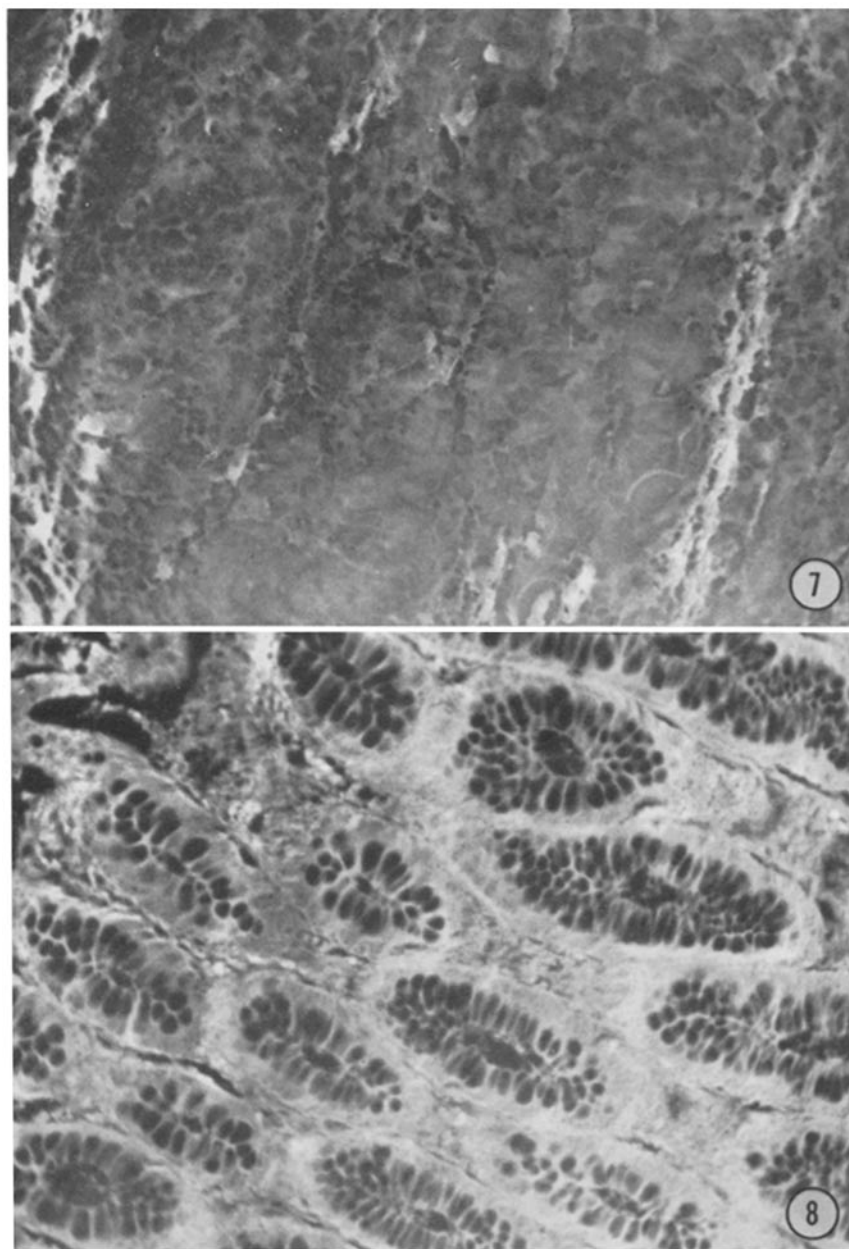
(Gold and Freedman: Tumor-specific antigens)

PLATE 38

FIG. 7. Section of tumor area treated with t_1-abs further absorbed with T_1. The absence of any significant fluorescence in this section indicates the specific inhibition of the tumor-specific antibody activity of t_1-abs by the tumor extract T_1. $\times$ 100.

FIG. 8. Section of normal colon treated with trs-150. Only a slight degree of fluorescein labeling is observed. $\times$ 100.
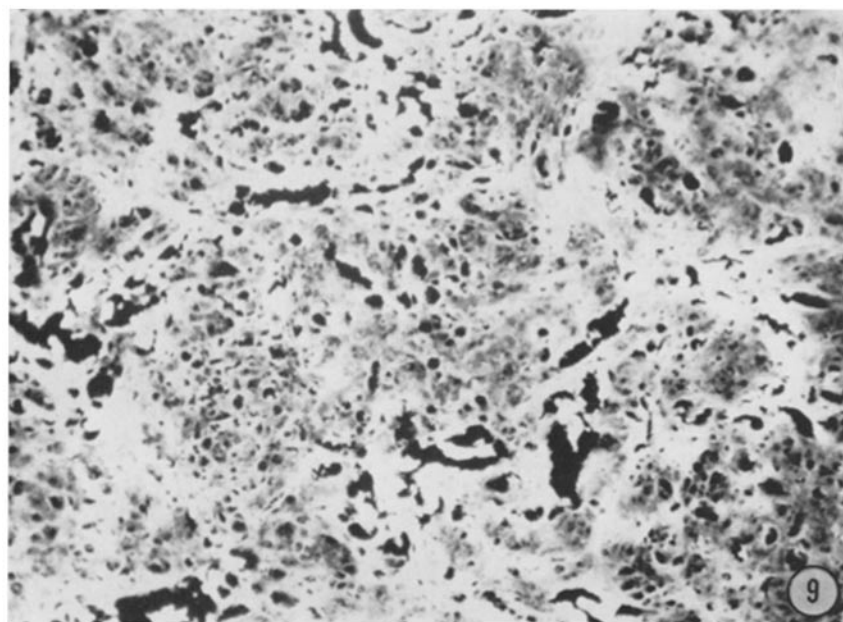
PLATE 38

(Gold and Freedman: Tumor-specific antigens)

PLATE 39

FIG. 9. Section of tumor area treated with trs-150. Intensity of fluorescence as compared with Fig. 8 indicates much greater antitumor than antinormal activity of trs-150. Comparison with Fig 6 suggests greater antitumor activity of trs-150 than t -abs. × 100.

(Gold and Freedman: Tumor-specific antigens)