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IMMUNOLOGICAL DIFFERENCES BETWEEN NORMAL AND MALIGNANT CELLS

BY

R. C. NAIRN, M.D., Ph.D.

H. G. RICHMOND, M.D.

M. G. McENTEGART, M.D.

AND

J. E. FOTHERGILL, Ph.D., B.Sc.

From the Departments of Pathology, Bacteriology, and
Biological Chemistry, University of Aberdeen

[WITH SPECIAL PLATE]

One of the traditional concepts of malignant change is that neoplastic cells lack certain growth-controlling enzyme systems present in normal cells. On this hypothesis, normal cells should contain proteins which are absent from malignant cells, and this difference in constitution should be demonstrable by immunological methods. Such an approach has been made by Weiler (1952), who compared the immunological properties of cytoplasmic particles from rat liver and from primary hepatoma induced by 4-dimethylaminoazobenzene (D.A.B.). He described an organ-specific antigen present in normal liver cells, but apparently absent from tumour. By means of the fluorescent antibody method he showed (1956a) that the livers of D.A.B.-treated rats developed islands of cells in which the antigen concentration was reduced in comparison with the surrounding parenchyma—that is, there was diminished staining of these islands in histological sections treated with specific anti-liver serum conjugated with fluorescein. The degree of antigen impoverishment varied with the total dose of carcinogen and the duration of the experiment. These findings indicated a correlation between loss of antigen and carcinogenic change. Similar observations were made in subsequent studies (1956b, 1956c) of stilboestrol-induced carcinoma in hamsters. The work, which has been recently reviewed (Weiler,

1959), has not, so far as we are aware, been confirmed by other workers, though an unsuccessful attempt to repeat it is reported by Hughes *et al.* (1957).

In the present investigation into the antigenic differences between normal tissues and tumours derived from them Weiler's findings in the D.A.B.-induced rat hepatoma have been corroborated. Similar results have also been obtained in preliminary studies of carcinoma in hamster kidney and of human skin tumours.

Methods

Liver Carcinoma in Rats

Production of Tumours.—The method was based on that of Griffin *et al.* (1948). Male albino rats (Tuck strain), weighing 200–250 g., were fed *ad lib.* for six months on a low-protein diet containing 0.06% 4-dimethylaminoazobenzene or for ten weeks on the low-protein diet containing 0.08% 3'-methyl-4-dimethylaminoazobenzene. When liver tumours became palpable the rats were killed by ether anaesthesia; thin blocks, each containing normal and tumour tissue, were "snap-frozen" in screw-cap bottles, pre-cooled in alcohol dry-ice freezing mixture, and stored at -20°C . Parallel blocks of tissue taken for conventional histological examination were fixed in formol-corrosive.

Preparation of Antigen.—Finely chopped fresh rat liver was suspended (10% w/v) in chilled 0.25 M sucrose buffered at pH 7.3 (0.1 M phosphate). The suspension was homogenized for four minutes at about 10,000 r.p.m. in an M.S.E. homogenizer. Further cellular disintegration, controlled by microscopical examination, was accomplished by means of the M.S.E.-Mullard ultrasonic disintegrator. A total of 90 seconds sonic treatment was given, resulting in breakdown of about 80% of cells with dispersal of cytoplasmic components; the majority of the nuclei remained intact. The cell components were separated as described by Weiler (1956a): 10 parts of the homogenate were layered on to one part of 10% sucrose solution, and centrifugation was carried out at 1,750 g for 7 minutes to remove tissue fragments and cell nuclei. The supernatant was then centrifuged at 9,000 g for 10 minutes to remove the mitochondrial sediment. Further centrifugation at 25,000 g for 90 minutes yielded cytoplasmic particles derived from the endoplasmic reticulum—that is, microsomal material, but presumably contaminated by fragments of cell membranes, nuclei, and mitochondria. This sediment was washed by resuspending in buffered sucrose followed by centrifugation, and the final sediment was snap-frozen in alcohol dry-ice freezing mixture and stored at -20°C . Weiler (1956a) showed that this final fraction provoked the best organ-specific antiserum, and it was therefore used as antigen throughout the following experiments.

Production, Absorption, and Testing of Antiserum.—The antigen was suspended in buffered saline (0.01 M phosphate, pH 7.1) at a concentration of 3.75 mg. antigen nitrogen per ml., and administered to three 2.5-kg. cross-bred female rabbits in 0.5-ml. doses intravenously at two- or three-day intervals for 24 days; the total dose of antigen N given to each rabbit was 18–19 mg. The animals were bled six days after the last injection. One rabbit produced a good antiserum with a complement-fixing titre of 1/320, and this was used in subsequent experiments; the titres in the other two rabbits were only 1/80 and 1/40 respectively. Complement-fixation tests were carried out with serial doubling

dilutions of serum tested against 7.5 γ antigen N; 3 M.H.D. of complement were used in the test and 2 M.H.D. in the controls. Fixation was carried out at 37° C. for 30 minutes, the haemolytic system added, and the results read after 30 minutes and 1 hour at 37° C. and after standing overnight at room temperature.

Immunological absorptions of the antiserum were carried out with acetone-dried tissue powders or with washed packed homogenates prepared from rat lung, kidney, liver, and hepatoma. The serum was shaken with powder (0.1 g. per ml.) or with homogenates (0.5 ml. per ml.) for 2 hours at room temperature; alternatively serum and homogenate were incubated at 37° C. for 30 minutes and allowed to stand overnight at 0° C. Serum was recovered, after absorption, by centrifugation at 10,000 g for 30 minutes. Successive absorptions were carried out in order to remove non-specific antibodies and leave only liver-specific antibody; this was removed by final absorption with liver powder or homogenate. The effect of successive absorptions was studied by (a) complement fixation; (b) gel diffusion by Mansi's (1958) modification of the Ouchterlony technique using 1.5 $\times$ 6 mm. wells 4 mm. apart; and (c) fluorescent antibody staining of tissue sections.

Staining with Fluorescent Antibody.—In order to obtain maximum staining sensitivity the "sandwich method" was adopted using labelled anti-rabbit-globulin serum prepared in a goat (G.A.R.G.), and the same general methods as described in a previous study (Nairn *et al.*, 1959). Direct staining was tried in preliminary experiments, but abandoned because the fluorescence with fully absorbed sera was too weak. Lissamine rhodamine B 200 (RB200) was used to prepare the fluorescent anti-rabbit-globulin conjugate (Chadwick *et al.*, 1958); it was absorbed with powdered activated charcoal (0.05 g. per ml. original serum), and then successively with rat liver powder, liver homogenate and lung homogenate, to eliminate non-specific staining. A few comparative experiments were also carried out with G.A.R.G. labelled with fluorescein isothiocyanate (Riggs *et al.*, 1958).

Frozen sections 5 μ thick were cut from the snap-frozen blocks of rat liver in a low-temperature freezing cabinet at -18° to -20° C., and mounted on gelatin-coated slides. After drying for 1 hour at 0° C. in a current of air, the sections were treated with absolute ethanol at -8° C. for five minutes and again dried in the cold. They were stained on the same or the following day after storage at 0° C., by applying a drop of the anti-rat-liver rabbit serum for 60 minutes in a damp container. After rinsing and washing for 15 minutes in three changes of buffered saline (pH 7.1) the sections were treated with conjugated G.A.R.G. for 20 minutes, washed twice for 10 minutes, and mounted in buffered glycerol. Control sections from rat kidney were also studied. Fluorescence microscopy was carried out with the Reichert equipment modified for ultra-violet blue light as described previously (Nairn *et al.*, 1958). Absence of staining in tissues treated with (a) normal (pre-immunization) rabbit serum, or (b) immune serum fully neutralized by absorption with liver preparations, provided tests of specificity.

Renal Carcinoma in Hamsters

Production of Tumours.—Renal carcinomas were induced in male golden hamsters by unilateral nephrectomy and subcutaneous implantation of a 15-mg.

pellet of hexoestrol (Horning, 1954). When a tumour became palpable the hamster was killed by ether anaesthesia and tissues were taken as described for the rat hepatoma.

Preparation of Antigen.—Normal hamster-kidney homogenates were prepared in the same way as the rat-liver homogenates and centrifuged at 2,000 g for 8 minutes to remove tissue fragments and cell nuclei. The supernatant (0.8 mg. N per ml.), consisting of the remaining cytoplasmic particles and soluble components, was used without further refinement for the production and testing of immune sera.

Production, Absorption, and Testing of Antiserum.—Different immunization procedures, in several rabbits, were used for the production of antisera. The best serum happened to be produced by an animal which had been given intramuscularly 5 mg. antigen N in Freund's adjuvant including mycobacteria, followed six months later by two booster courses each of three daily injections of 0.8 mg. antigen N with an interval of six weeks between courses; the animal was bled five days after the last injection. This serum had a complement-fixing titre of 1/160 when tested against 16 γ antigen N. It gave four lines against the antigen in gel-diffusion tests. Successive absorptions were carried out with packed sheep red cells to remove Forssman antibody, and with powders or homogenates prepared from hamster lung, liver, and renal carcinoma to remove non-specific antibodies and leave only kidney-specific antibody; this was removed by final absorptions with kidney powder and homogenate. As in the rat-liver studies, the effect of successive absorptions was analysed by complement fixation, gel diffusion, and fluorescent antibody staining. Sections of hamster kidney, renal carcinoma, and liver were stained by the same procedure as before; the conjugated G.A.R.G. was absorbed with activated charcoal, hamster liver powder, and homogenates of hamster lung and kidney.

Skin Tumours in Man

Representative blocks, including normal skin and tumour, were taken from a variety of human skin tumours, snap-frozen and stored at -20° C. Eighteen tumours were studied: 5 squamous carcinomas, 5 basal-cell carcinomas, 2 naevocarcinomas, 1 sweat-gland carcinoma, 2 papillomas, 2 benign naevi, and 1 benign sweat-gland tumour. Other blocks examined were from normal skin, keratotic lesions, 1 leucoplakia of lip, and 2 squamous carcinomas of oesophagus.

Preparation of Antigen.—Preliminary experiments had shown that skin is extremely resistant to disintegration by standard methods, and best results were obtained by the following procedure. Skin, taken with a Humby knife from the thigh of an adult man six hours after death, was finely chopped, suspended in buffered saline (0.01 M phosphate, pH 7.1), and treated in the homogenizer at 10,000 r.p.m. for 4 minutes. The resulting coarse homogenate was forced, frozen at -20° C., through a Hughes press (Hughes, 1951). The partially disintegrated skin was again homogenized at 10,000 r.p.m. and then given 3 minutes' sonic treatment. Though these procedures disrupted sufficient cells to provide the cytoplasmic components for preliminary studies, many intact cells and islands of squamous epithelium remained. Sucrose was added to the homogenate to make a 0.25 M solution, and tissue fragments and cell nuclei were removed by centrifugation

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FIG. 1

FIG. 4

FIG. 2

FIG. 5

FIG. 3

FIG. 6

FIG. 7

at 2,000 g for 8 minutes; the supernatant (0.4 mg. N per ml.) was used as skin antigen without further refinement.

Production, Absorption, and Testing of Antiserum.—There was only enough antigen to immunize one rabbit, which was given initially 4 mg. antigen N in Freund's adjuvant, including mycobacteria, intramuscularly, and six weeks later a booster course of three daily intraperitoneal injections of 0.8 mg. antigen N. At further intervals of four weeks the booster course was repeated twice; the animal was bled five days after the last injection. There was insufficient antigen for complement-fixation tests, and the serum was therefore studied by gel diffusion and fluorescent-antibody staining. Sections of human skin, skin tumours, liver, and kidney were studied; the conjugated G.A.R.G. was absorbed with activated charcoal, human liver powder, and homogenates of human lung and skin.

Results

Liver Carcinoma in Rats

The effects of the various immunological absorptions on the anti-rat-liver serum are summarized in Table I. A fall in complement-fixing titre was often observed when tests were repeated, due, we believe, to some impairment of the antigen during storage, freezing, and thawing; where this has occurred a range of titres is given. Despite these variations, there was progressive

loss of titre with successive absorptions: after absorbing first with lung homogenate, secondly with kidney powder, and thirdly with kidney homogenate, the titre fell from 1/320–1/160 to 1/20–1/5; when tumour homogenate instead of kidney homogenate was used for the third absorption the titre fell to 1/5–1/2.5; there was complete loss of titre when liver homogenate was used for the third absorption. The same results were obtained in a few parallel experiments when, instead of homogenates, tumour powder and liver powder respectively were used for third absorptions.

A similar progressive loss of antibody components was observed when sera from successive absorptions were tested against the liver antigen by gel diffusion. The production of specific precipitin lines by this technique indicates that the antigen suspension was partially soluble. Unabsorbed serum gave three specific lines, and serum absorbed with lung homogenate gave two lines; after absorption of this serum with kidney powder and homogenate two lines (one faint) could still be obtained. When tumour homogenate was used for the third absorption the serum gave a single line; there was no line when liver homogenate was used for this absorption. Normal serum, unabsorbed or absorbed, gave no lines.

The fluorescent-staining reactions of sections of rat liver and tumour and of normal rat kidney are also shown in Table I. Twenty tumour blocks obtained from six rats were studied; they included hepatomas, cholangiomas, and mixed types, and showed a wide range of differentiation and anaplasia. The table presents a composite picture of the staining obtained by applying the RB200-conjugated G.A.R.G. after treatment of the sections with the normal and immune sera with and without absorptions. The unabsorbed anti-liver serum gave strong diffuse staining of liver parenchyma and of tumour, but less staining of normal rat kidney. After a single absorption with lung homogenate the staining was more clearly defined, and obviously more intense in liver than in tumour and least intense in kidney. Further absorptions with kidney powder and homogenate abolished kidney staining by the serum, but staining could still be obtained in liver and to a minor extent in tumour. When tumour homogenate was used for the third absorption of the serum, staining was diminished in the liver and virtually absent in the tumour; there was no staining when liver homogenate was used for the third absorption. Normal serum gave slight, diffuse, non-specific staining equally in liver, tumour, and kidney, and no staining after absorption with rat-tissue preparations. Some of the liver sections contained hyperplastic, possibly pre-neoplastic, nodules of liver cells, which were stained by the antiserum with an intensity somewhere between the staining of normal liver and of carcinoma.

The localization of specific staining by highly absorbed sera in the liver parenchyma was mainly in the cell membrane and in the perinuclear zone, with faintly staining granules elsewhere in the cytoplasm; occasionally the cytoplasmic staining appeared to be diffuse rather than granular (Special Plate, Figs. 1 and 2). Staining of tumour cells by less fully absorbed sera was more diffuse, though the perinuclear component could usually be distinguished. Incompletely absorbed sera gave some staining of connective tissue and conspicuous staining of vascular lining in liver, tumour, and kidney; staining of renal tubular epithelium was minimal even with unabsorbed serum.

DESCRIPTION OF SPECIAL PLATE

All except Figs. 2 and 5 are fluorescence photomicrographs of frozen sections stained by "sandwich method" with RB200-conjugated goat anti-rabbit globulin after treatment with absorbed anti-organ rabbit sera. The large bright spot in Figs. 3, 4, and 6 is an artifact used as an aid to focusing.

FIG. 1.—Rat-liver section treated with anti-rat-liver serum which had been absorbed successively with rat lung homogenate, kidney powder, and kidney homogenate. Fluorescent staining is brighter in the normal parenchyma, to the left, than in the carcinoma, to the right. The diffuse appearance of the fluorescence is due in part to limitations of the photomicrography. ($\times 120$.)

FIG. 2.—Parallel section to Fig. 1 fixed in formol-acetate and stained with haematoxylin and eosin. Between the normal and carcinoma there is a necrotic zone infiltrated with leucocytes. ($\times 120$.)

FIG. 3.—Normal hamster-kidney section treated with anti-hamster-kidney serum which had been absorbed successively with hamster-lung homogenate, sheep red cells, hamster-renal-tumour powder and homogenate. There is fluorescent staining of cell membrane of renal tubular cells, particularly of the brush border of a proximal convoluted tubule, top left; crescentic perinuclear staining and a few fluorescent cytoplasmic particles can also be seen. ($\times 830$.)

FIG. 4.—Human squamous carcinoma section treated with anti-human-skin serum which had been absorbed successively with human lung homogenate, liver powder, and liver homogenate. Normal epidermis, above, has stained more brightly than tumour, below. ($\times 180$.)

FIG. 5.—Same field as Fig. 4 after staining with haematoxylin and eosin, showing normal epidermis with underlying poorly differentiated carcinoma. ($\times 180$.)

FIG. 6.—Human naevocarcinoma treated with same serum as in Fig. 4, showing the same contrast between normal epidermis and underlying tumour; aggregates of dimly staining naevocarcinoma cells can also be seen in transected dermal papillae. ($\times 120$.)

FIG. 7.—Human basal-cell carcinoma section treated with same serum as in Figs. 4 and 6. Bright fluorescent staining of cell membrane is well shown in normal epidermis, above, and contrasts with dimmer staining of the tumour cells, below. ($\times 550$.)

The histological findings were similar in the few experiments in which G.A.R.G. conjugated with fluorescein was used, but, owing to lack of colour contrast with tissue autofluorescence, definition was poorer and localization to the cell membranes less conspicuous. Photomicrographs of such preparations may be misleading, since the autofluorescence of normal liver is usually brighter than that of carcinoma, and thus augments the difference in fluorescence between the two tissues when they are stained with a fluorescein conjugate.

The three methods of immunological analysis show parallel changes in the anti-rat-liver serum after successive absorptions. The results, in particular the gel-diffusion analysis, suggest that the antigen contains at least three distinct components. Two of these are present in tissue other than liver and are therefore not organ-specific. The third component appears to be organ-specific; it may or may not be exclusive to liver, but is undoubtedly present in much higher concentration in the liver than in the other tissues used for the serum absorptions. The precipitin line produced by the tumour-absorbed antiserum could still be obtained after reabsorption of the serum with tumour homogenate.

Thus it would seem that the liver-specific component of the antigen is either absent altogether from the liver tumour or at most present in very small amount.

Renal Carcinoma in Hamsters

The reactions between hamster tissues and the anti-hamster-kidney serum after successive absorptions showed the same general pattern (Table II) as in the rat-liver experiments. Absorption of the antiserum with hamster-lung homogenate to remove species-specific antibodies to hamster tissue, in particular reticulin, and with sheep red cells to remove Forssman antibody, resulted in a fall in the complement-fixation titre from 1/160 to 1/10. The titre remained at 1/10 after two further absorptions with liver powder and homogenate or with renal tumour powder and homogenate. Kidney powder and homogenate caused a reduction in titre, possibly complete loss, but this could not be measured because we were unable to overcome the anticomplementary activity of the serum after these absorptions.

Parallel changes occurred in the gel-diffusion tests: serum before absorption gave four lines with the kidney antigen and two lines (one faint) after absorptions with lung homogenate and sheep red cells. The strong line

TABLE I.—Reactions of Unabsorbed and Absorbed Anti-rat-liver Rabbit Serum

Absorptions with Rat-tissue Preparations:	Anti-rat-liver Serum					Normal (Pre-immunization) Serum	
	Nil	Lung Homog.	Lung Homog. + Kidney Powder + Kidney Homog.	Lung Homog. + Kidney Powder + Tumour Homog.	Lung Homog. + Kidney Powder + Liver Homog.	Nil	Lung Homog. + Kidney Powder + Kidney Homog.
Complement-fixation titre against 7.5 γ antigen N*	1/320-1/160	1/80	1/20-1/5	1/5-1/2.5	0	0	...
Gel-diffusion lines at 48 hours against antigen (3.75 mg. N per ml.)	3	2	2† (1 faint)	1†	0	0	0
Staining of rat tissues with fluorescent antibody ‡							
Liver { normal	++++	+++	+(+)	+	—	(+)&	—
tumour	++++	++	(+)	(-)	—	(+)&	—
Kidney—normal	+++	+	(-)	(-)	—	(+)&	—

(+) = Minor staining. (-) = Minimal patchy staining sometimes seen. * Range given where repeated tests differed in titre. † One line still obtained after further absorption with kidney or tumour homogenate. ‡ Composite picture from all experiments. § Diffuse non-specific staining.

TABLE II.—Reactions of Unabsorbed and Absorbed Anti-hamster-kidney Rabbit Serum

Absorptions with Hamster-tissue Preparations:	Anti-hamster-kidney Serum					Normal (Pre-immunization) Serum	
	Nil	Lung Homog. + Sheep Red Cells	Lung Homog. + Sheep Red Cells + Liver Powder and Homog.	Lung Homog. + Sheep Red Cells + Tumour Powder and Homog.	Lung Homog. + Sheep Red Cells + Kidney Powder and Homog.	Nil	Lung Homog. + Liver Powder
Complement-fixation titre against 16 γ antigen N	1/160-1/80	1/10	1/10	1/10	Anticomplementary < 1/10	0	...
Gel-diffusion lines at 48 hours against antigen (0.8 mg. N per ml.)	4	2 (1 faint)	1	1	0	0	0
Staining of hamster tissues with fluorescent antibody:							
Kidney { normal	++++	+++	+++	+++	(+)	(+)	—
tumour	++++	+++	+(+)	+	(+)	+	(+)
Liver—normal	++	+(+)	(-)	(-)	(-)	(+)	—

TABLE III.—Reactions of Unabsorbed and Absorbed Anti-human-skin Rabbit Serum

Absorptions with Human-tissue Preparations:	Anti-human-skin Serum				Normal (Pre-immunization) Serum	
	Nil	Lung Homog. + Liver Powder	Lung Homog. + Liver Powder + Liver Homog.*	Lung Homog. + Liver Powder + Skin Homog.*	Nil	Lung Homog. + Liver Powder + Liver Homog.
Gel-diffusion line at 48 hours against antigen (2 mg. N per ml.)	3	1	1	1 faint†	0	0
Staining of human tissues with fluorescent antibody:						
Skin { normal, hyperplastic, and papilloma	+++	+++	++	(-)	(+)	—
carcinoma	+++	++	+	(-)	(+)	—
Kidney and liver—normal	++	+	—	—	(+)	—

* The results were the same whether the serum was absorbed once or twice with these homogenates. † Abolished by treatment with freeze-dried soluble material from skin homogenates.

could still be obtained after two further absorptions of the serum with liver or tumour preparations, but there were no lines when kidney preparations were used for these absorptions. Normal serum gave no lines. The results suggest that the antigen component responsible for the single line against the highly absorbed serum is kidney-specific and is absent from the tumour tissue.

This conclusion is supported by the fluorescent-staining reactions. Four tumour blocks from two renal carcinomas were studied; the staining was compared with that of the adjacent normal kidney and with normal kidney and liver blocks from other hamsters. The reduction of staining in kidney, tumour, and liver, after successive absorptions of the antiserum, followed the same pattern as the other immunological tests. The difference in staining between the three tissues also fits with the rest of the findings: with unabsorbed serum staining was intense and diffuse in kidney and tumour, and less intense in liver; after absorption with lung and sheep red cells the serum gave brilliant, clearly defined staining of kidney and tumour and less staining of liver. Further absorptions by liver or tumour preparations did not diminish the staining of kidney, though staining was reduced in tumour and virtually abolished in liver; there was little or no staining after the absorptions with kidney preparations. The normal sera gave unusually bright, diffuse staining of tumour, presumably a non-specific physical effect. The specific staining of kidney produced by the highly absorbed sera was located mainly in the tubular epithelial cells in cell membrane, the perinuclear zone, and in a few cytoplasmic granules (Special Plate, Fig. 3); there was also some glomerular staining. Staining of tumour by the highly absorbed sera was confined to the cell membrane. With incompletely absorbed sera there was more granular cytoplasmic staining, less prominent in tumour than in normal kidney tubules. Unabsorbed sera also gave staining of vascular lining and connective tissue in kidney, tumour, and liver.

Skin Tumours in Man

The results of fluorescent-antibody staining of human tumours (Table III) were strikingly similar to those obtained with the animal tumours. The unabsorbed serum gave strong diffuse staining of normal skin and all skin tumours, and less staining of control sections of normal human kidney and liver. After absorption of the serum with lung homogenate and liver powder the staining of normal skin was just as bright, more clearly defined, and limited to the epidermis; there was much less staining of the control sections. After further absorption with liver homogenate the difference was even more obvious. Thus it was clear that this highly absorbed serum had selective affinity for skin; the same affinity was shown for benign tumours of skin—that is, there was no difference between the staining of these tumours and of the adjacent normal epithelium. Malignant skin tumours, however, stained less intensely than normal epithelium (Special Plate, Figs. 4–7). All staining was virtually abolished when skin homogenates were used for the final absorption. For effective absorption the skin homogenates required the same drastic methods of preparation as previously employed for the production of skin antigen; less disrupted preparations were ineffective.

The specific staining of epidermis by the highly absorbed sera was largely confined to the cell membranes, and was more intense in the basal-cell than the prickle-cell layer; some cytoplasmic granular staining

was also present. The same pattern and degree of staining were observed in hyperplastic papillae of the keratotic and leukoplakic lesions and in the papillomas. Staining was diminished in 12 of the 13 carcinomas (a naevocarcinoma was the only exception); it was confined to cell membrane, and contrasted sharply with the bright staining of the adjacent normal epidermis. The extra fluorescent staining seen in the basal-cell layer of normal epidermis had no counterpart in the peripheral-cell layer of tumour masses in the dermis. The oesophageal carcinoma also showed reduced staining in the islands of cancer cells when compared with the adjacent normal and hyperplastic epithelium.

The changes observed when the sera from successive absorptions were tested by gel diffusion were less systematic. In order to obtain satisfactory lines the antigen was concentrated to 2 mg. N per ml.; it gave three lines against the unabsorbed serum, and one sharp line after absorption of the serum with lung homogenate and liver powder. A sharp line could still be obtained after further absorption with liver homogenate, but only a faint line was produced when skin homogenate was used for the absorption. This faint line could also be demonstrated after additional absorption with sheep red cells or human red cells (Group A, B, or O), but not after treatment with freeze-dried soluble material from skin homogenates. Failure to absorb the serum completely with washed skin homogenates is perhaps explained by the difficulty of making such preparations satisfactorily, and this problem was never entirely overcome. Various means, such as the preparation of squamous-epithelial-cell homogenates from microtome sections of oesophageal mucosa, were only partially effective. Scarcity of antiserum precluded further attempts to solve the problem, and we were unable to study parallel absorptions by normal skin and skin-tumour preparations.

The fluorescent-staining experiments suggest that the antiserum contains specific antibody to antigen present in normal skin epidermis and in benign skin tumours; in malignant tumours the amount of antigen is reduced. This antigen may be responsible for the single precipitin line obtained with the absorbed sera, but the evidence is not conclusive.

Discussion

These findings suggest that mammalian cells contain organ-specific antigen or antigens which are lost or depleted when the cells become malignant, whether the malignancy is chemically induced or spontaneous. The results are most clear-cut in the rat-liver study, possibly because the antigen was less crude than in the other investigations. Nevertheless, all three studies gave consistent results, especially with the fluorescent-antibody method. In normal rat liver, hamster kidney, and human epidermis staining was strongest in the cell membranes. This localization is not described in Weiler's studies; he used fluorescein-labelled antibody for staining, and cell-membrane staining is less conspicuous with this method than with RB200 labelling. Affinity of the specific antiserum for cell membrane is not unexpected; our antigens contained endoplasmic reticulum, which may have morphological continuity with cell membrane (Robertson, 1959), and hence there might well be antigenic components common to both structures. Cytoplasmic staining, seen best in the rat liver, was less obvious in hamster kidney and least in human skin. This variation is not necessarily due to fundamental differences in the tissues themselves, but

may merely reflect technical differences in the production of the antigens and hence the antisera.

Apart from the visual information from fluorescent staining, the principal evidence for the organ-specificity of the rat-liver and hamster-kidney antisera is the failure to abolish immunological activity except by absorptions with rat-liver or hamster-kidney preparations respectively. The persistence of complement-fixing activity and the production of a single precipitin line against the antigen, by sera repeatedly absorbed with other tissues, support this view. The general pattern of results with the human-skin antiserum follows the animal studies so closely that it seems reasonable to regard the serum as organ-specific. The evidence for antigen depletion in the tumours compared with the normal tissues is provided by their differential staining with labelled antiserum and their differential antibody-neutralizing properties. The depletion may be absolute in the experimental carcinomas, but appeared to be only partial in human skin cancer. Hiramoto and Pressman (1957), in a brief report of fluorescent-staining reactions by an anti-human-skin serum, mention selective staining of skin tumours by the serum, but not differential staining.

There is close correspondence between our results and those of Weiler, and the failure of Hughes *et al.* to produce a rat-liver-specific antiserum is difficult to understand. These authors infer that the differential staining of normal tissue and tumour demonstrated by Weiler may have been a non-specific reaction, but this interpretation ignores the careful controls in Weiler's experiments; similar controls have also been used in the present studies. We (Nairn *et al.*, 1960) have examined the kind of non-specific staining described by Hughes *et al.*, and conclude that it is different in kind and degree from that produced by organ-specific antisera.

What bearing has the loss of organ-specific antigen on carcinogenesis? The protein-deletion theory (Miller and Miller, 1952) postulates that the binding of carcinogens to growth-controlling cell proteins leads to inactivation of these proteins, which thus fail to be replicated in subsequent cell divisions. The daughter cells are neoplastic because they lack the growth-control factor. Vogt (1958, 1960) has shown that the organ-specific antigen in rat liver is found in the membranes of the endoplasmic reticulum, and he suggests that degradation of these membranes in the course of carcinogenesis contributes to the uncontrolled activity of the cancer cell. The site and the organ-specificity of the antigen loss may, however, have other implications. Green (1959) has suggested that growth of normal cells is controlled immunologically by virtue of the presence of specific lipoproteins; absence of such "self markers" permits neoplastic behaviour. As it is reasonable to suppose that the organism may identify its cells by their surface structure, then our observation that the cell membrane is an important site of organ-specific antigen may be significant.

Summary

An alteration in cell constituents associated with malignant change has been investigated by immunological methods. Three types of normal and tumour tissue were compared: rat liver and hepatoma (induced by aminoazo-dye feeding); hamster kidney and renal carcinoma (induced by oestrogen implant); human skin and various skin tumours (occurring spontaneously in man). In each study antigen was prepared from homogenized normal tissue, which was centrifuged to remove

tissue fragments and cell nuclei. For the hamster-kidney and human-skin experiments rabbits were immunized with the crude supernatants; for the rat-liver study the homogenate was further refined so that the antigen used for immunization consisted mainly of cytoplasmic particles derived from the endoplasmic reticulum—that is, microsomal material.

The rabbit antisera were tested against the corresponding normal and tumour tissues by three methods, using appropriate tissue preparations to absorb and remove non-organ-specific antibodies: (1) complement fixation; (2) gel-diffusion analysis; and (3) fluorescent staining by the "sandwich" technique. Control studies were carried out with normal (pre-immunization) serum and immune serum specifically neutralized by absorption with preparations of the homologous organ. In the rat-liver and hamster-kidney experiments all three methods indicate the presence of an organ-specific antigen in liver and kidney respectively; the antigens are either absent from, or at most present in very small amount in, the corresponding tumour. In the malignant tumours of skin a partial depletion of specific antigen was demonstrated; technical difficulties in preparing skin and skin-tumour homogenates for control absorptions prevented more complete analysis. The fluorescent-antibody studies suggest that the specific antigen is localized in the cell membrane and in the perinuclear zone, with some faintly staining granules elsewhere in the cytoplasm.

One interpretation of these findings is that the lack of organ-specific antigen may indicate absence of a particular growth-controlling factor from malignant cells. The localization of the antigen to normal cell membrane may have special significance and be related to surface identification of the cell. Controlled normal growth might require such a "self-marker," and its absence would lead to uncontrolled malignant growth.

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