DIFFERENCES IN STAINING OF NORMAL AND MALIGNANT CELLS BY NON-IMMUNE FLUORESCENT PROTEIN CONJUGATES

BY

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

H. G. RICHMOND, M.D.

AND

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

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

Differential staining of normal and malignant tissue by normal serum globulin labelled with fluorescein was described in 1957 by Hughes, Louis, Dineen, and Spector. In the course of an attempt to repeat Weiler's (1956) fluorescent antibody studies of rat hepatoma, they found that labelled non-immune rabbit globulin (N.R.G.) stained normal liver but not tumour, and that the fluorescent staining appeared to be similar to that obtained by Weiler with labelled organ-specific anti-serum. The staining reaction with non-immune serum has been observed in human and experimental material and has been produced with different serum-protein fractions from several animal species (literature quoted by King, Hughes, and Louis, 1958). It has been suggested that the reaction might provide a simple histological test of malignancy.

In the present study the staining method with non-immune conjugates has been applied to a variety of tumours, human and experimental, and our results do not show a uniform pattern. Normal tissue stained more brightly than tumour in some cases, particularly in colonic carcinoma, but in others there was no differential staining and occasionally the normal tissue stained less brightly than tumour. In a few experiments our histological findings have been compared with the results of centrifugation and electrophoresis of mixtures of conjugated N.R.G. with homogenates of normal and tumour tissue. This comparison suggests that the soluble rather than the particulate components of the cells are concerned in the non-specific staining.

Methods

Human tumours were collected mainly from operation specimens immediately after resection, and occasionally from necropsies when death had occurred less than eight hours previously. Blocks containing both tumour and normal tissue were "snap-frozen" in screw-cap bottles pre-cooled in alcohol-dry ice mixture, or were frozen in a tube of isopentane immersed in the freezing mixture. The following types of tumour were studied:—*Skin*: squamous carcinoma; basal-cell carcinoma; naevocarcinoma; sweat-gland carcinoma; papilloma; benign naevus; benign sweat-gland tumour. *Oesophagus*: squamous carcinoma. *Stomach*: spheroidal-cell carcinoma; adenocarcinoma. *Colon*: adenocarcinoma; benign polyp. *Kidney*: hypernephroma. *Breast*: carcinoma; fibroadenoma. *Pancreas*: carcinoma. *Salivary gland*: mixed tumour. Some hyperplastic, possibly preneoplastic, conditions were also examined—namely, cystic epithelial hyperplasia of breast, and keratotic and leucoplakic lesions of skin.

Animal tumours from rat liver and hamster kidney, as in the preceding paper (Nairn, Richmond, McEntegart, and Fothergill, 1960), were also used in the present investigation.

Histological Studies

For section cutting and staining, the same general methods as in the preceding paper were used. Frozen sections, 5 μ thick, mounted and dried, were treated with absolute ethanol at -20° C. for 15 minutes and again dried at 0° C. They were stained for 10 minutes with N.R.G. conjugated with lissamine rhodamine B 200 (RB200); after rinsing and washing for 10 minutes in two changes of buffered saline (0.01 M phosphate, pH 7.1) they were mounted in buffered glycerol and examined by fluorescence microscopy. In order to compare the results with those of King *et al.* (1958), a parallel series of experiments was carried out using the same methods (Hughes, 1958) with fluorescein conjugates, prepared from fluorescein isocyanate derived from isomer I aminofluorescein (Coons and Kaplan, 1950). The conjugates were extracted with ethyl acetate (Dineen and Ada, 1957), a method which leaves a substantial proportion of unreacted fluorescent material (U.F.M.) adsorbed on the protein.

The possible influence of such U.F.M. on staining was studied in a further series of experiments using fluorescein conjugates treated with activated charcoal, which removes the U.F.M. almost completely (Chadwick and Nairn, 1960). Other staining experiments were carried out with an aqueous solution of U.F.M. prepared by a mock conjugation procedure in which fluorescein isocyanate was mixed with carbonate-buffered saline (pH 9) but without the addition of serum protein. This mixture was stirred overnight, and then diluted with a large volume of phosphate-buffered saline (pH 7.1) to make a solution of the same fluorescence intensity as the fluorescein-conjugated N.R.G. which had been extracted with ethyl acetate. The nature and staining properties of this U.F.M. solution are discussed elsewhere (Chadwick and Nairn, 1960). For comparison with the fluorescent staining, sections treated with brilliant cresyl blue, haematoxylin and eosin, and methyl-green-pyronin were also examined.

Centrifugation and Electrophoretic Studies of Tissue Homogenates

Normal and carcinoma tissue from human colon (one post-mortem and five surgical specimens), human kidney (one post-mortem and one surgical specimen), and rat liver (one specimen) were homogenized in 3 vols. buffered saline (pH 7.1) containing penicillin and streptomycin (500 units/ml. of each) by means of an M.S.E. homogenizer at 10,000 r.p.m. for four minutes. The homogenates were diluted to 10% (w/v) and further cellular disintegration was accomplished by means of the M.S.E.-Mullard ultrasonic disintegrator. Tissue homogenates and RB200-conjugated N.R.G. were mixed in the ratio 30 parts tissue protein nitrogen to 1 part N.R.G. nitrogen and allowed to stand at 0° C. for 30 minutes before centrifuging at 105,000 *g* for 45 minutes; appropriate blanks were run in parallel. In early experiments the medium included sucrose (0.25 M), but this had no effect on the results and was omitted subsequently.

Spectrophotometric optical density measurements at 565 $m\mu$ were carried out on the supernatants to determine if any of the conjugate had been absorbed by the subcellular particles. These supernatants, presumably mainly protein in solution, were not fluor-

escent enough for satisfactory electrophoretic study, and therefore they were concentrated by evaporation, dialysed against 0.2 M borate buffer at pH 8.6 (Adjutantis, 1954), and adjusted to a protein concentration of 10 mg./ml.; more RB200-conjugated N.R.G. was added to make mixtures of equal parts of N.R.G. and tissue protein. Electrophoresis was carried out in the borate buffer, using an E.E.L. apparatus; 20 μ l. of each fluorescent mixture was applied to a 34 by 5 cm. strip of Whatman No. 1 filter paper and a current of 1.5 mA passed for 16 hours. Dried filter-paper strips were examined under ultra-violet light, and a visual estimate was made of the intensity of fluorescence on the starting-line. This was compared with that of a control run with RB200-conjugated N.R.G. alone, most of which remains on the starting-line at pH 8.6. The difference gave an indication in each of the fluorescent mixtures, of the proportion of RB200-conjugated N.R.G. which had migrated in association with the tissue proteins.

Results

In the accompanying Table the staining with RB200-conjugated N.R.G. of 65 human and 8 animal tumours is compared with that of the adjacent normal tissues.

Comparison of Fluorescent Staining of Normal and Tumour Tissue by Normal Rabbit Globulin Conjugates

	Comparative Staining		
	Normal > Tumour	Normal = Tumour	Normal < Tumour
Human carcinomas:			
Skin (18)	7	11	
Oesophagus (2)	1	1	
Stomach (6)	3	2+1*	
Colon (14)	12	1+1*	
Kidney (5)		1+2*	2*
Breast (6)	2	3	1
Pancreas (1)	1		
Animal carcinomas:			
Rat liver (6)	1	5	
Hamster kidney (2)	1		1
Human benign tumours:			
Skin (8)	1	7	
Colon (3)		2+1*	
Breast (1)			1
Salivary gland (1)			1
Total (73)	29	38	6

* Necropsy material.

Only the human colon carcinomas showed a definite trend of brighter staining in the normal mucosa compared with tumour (12 out of 14 cases); two cases showed equal staining, but one of these was necropsy material. Almost the opposite result was obtained in the five renal carcinomas, four of which were necropsy material: two showed brighter staining of tumour and three equal staining of tumour and normal. One other human carcinoma, from the breast, showed brighter staining of tumour. The carcinomas of the skin, oesophagus, stomach, and pancreas showed equal or less staining when compared with the normal tissues. Five rat hepatomas showed equal staining with the normal rat liver and one showed diminished staining. One hamster kidney tumour stained less brightly and the other more brightly than the normal kidney. One of 13 benign tumours stained less, 10 equally, and 2 more brightly than the corresponding normal tissue. The hyperplastic lesions (one case of cystic epithelial hyperplasia of breast, one leucoplakia of lip, and three keratoses of skin) showed equal staining in normal and abnormal tissue.

Many other tumours and hyperplastic lesions were examined, but staining comparisons could not be made

because the tissue blocks did not include identifiable samples of both normal and abnormal tissue. This sampling problem was particularly troublesome with breast tumours, in which it was always difficult to include representative normal acini in the same block as tumour. For all practical purposes our results were the same whether sections were taken immediately from tissue blocks frozen in isopentane or from stored snap-frozen material, and whether staining was carried out with RB200-conjugated N.R.G. or with fluorescein conjugates. Assessment of the degree of staining by fluorescein conjugates required the examination of parallel unstained sections to allow for the effect of autofluorescence; this is usually brighter in normal than in carcinoma tissue, partly because of a greater concentration of non-fluorescent nuclear material in tumour.

These control studies are of particular importance since colour contrast is poor between the green of fluorescein conjugates and blue-green of autofluorescence, and the staining was often dim and diffuse. The orange fluorescent staining with RB200-conjugated N.R.G. was easier to assess because of better contrast with autofluorescence. Another difficulty in the interpretation of staining of the carcinomas was the frequent occurrence of areas of necrosis which not only had different autofluorescence from the rest of the tissue but showed inconsistent staining reactions: fluorescent staining of necrotic areas was usually weak, but occasionally it was intense.

Staining by fluorescein conjugates extracted with ethyl acetate was brighter than with charcoal-treated conjugates, presumably because of a contribution from U.F.M. adsorbed on the serum globulin. The influence of this U.F.M., which probably comprises aminofluorescein and another fluorescent product of hydrolysis of fluorescein isocyanate, was studied in parallel experiments. Staining with aqueous U.F.M. solution was more brilliant than with conjugated N.R.G.: its distribution was more often equal in the tumour and normal tissues (in 46 of the 73 tumours); six carcinomas (three skin, one stomach, one colon, and one breast) gave greater staining in the normal tissue, though staining with N.R.G. conjugates was equal in tumour and normal.

The intracellular distribution of staining by the fluorescent conjugates was usually diffuse in the cytoplasm except in normal colonic mucosa cells, which showed sharp peripheral staining and clearly outlined non-fluorescent vacuoles. Some degree of staining was seen in all tumours and corresponding normal tissues; this is contrary to the experience of King *et al.*, who record no staining in malignant tumours. In all tissues, staining was commonly dim, and differences between tumour and normal were often so small that time and histological judgment were required for any distinction to be made. No correlation was observed between the intensity or distribution of the fluorescent staining and the ribonucleic acid content of the cells as indicated by the pyronine reaction. There was, however, close correlation with the acidophilic—for example, eosinophilic—cytoplasmic staining; basophilic cells showed less affinity for the fluorescent conjugates. The histological findings suggest, therefore, that this non-specific fluorescent staining of normal tissue and tumour by N.R.G. conjugates is a property of cytoplasm similar to that demonstrated by conventional staining methods. This view is supported by the staining results with the

U.F.M. solution, which, from its probable chemical composition, would be expected to behave like eosin.

Studies of Tissue Homogenates.—In the nine experiments comparing the affinities of RB200-conjugated N.R.G. for normal and tumour homogenates, the optical densities of the supernatants after centrifugation showed no loss of conjugate—that is, there was no apparent absorption of conjugate by the sedimented material. The method is sensitive enough to detect a 10% absorption of conjugate which would represent 1 part of conjugate to 200 parts of sediment. Visual comparison of the electrophoretic strips from the normal and tumour supernatants showed greater affinity of the conjugate for normal than for tumour protein in four samples of human colon carcinoma—that is, there was less fluorescence on the starting-line of the normal sample. Approximately equal affinities were observed in two cases of human colon carcinoma, in one human renal carcinoma, and in the rat-liver carcinoma. The other human renal carcinoma showed greater affinity for the conjugate than did the corresponding normal material. The soluble tissue proteins appeared to bind roughly half their own weight of RB200-conjugated N.R.G. and could bind all the conjugate when they were present in excess (4 parts tissue protein to 1 part conjugate). All the results of electrophoresis agreed with the histological staining reactions by the conjugated N.R.G. Because the homogenate studies were carried out on “unfixed tissue,” unfixed sections of the corresponding tissue blocks were examined in parallel with the usual “fixed” (alcohol-treated) material: fixation had no obvious effect on the staining apart from occasional improvement of histological definition.

These results suggest that the soluble cytoplasmic components are concerned in the non-specific fluorescent staining of normal and tumour tissue.

Discussion

The trend of these results is towards equal staining of normal tissue and benign tumours, and diminished staining of carcinomas. Although we have followed the published methods of King *et al.* with great care, we have been unable to achieve their decisive results. While the trend we have observed points to an interesting cytoplasmic difference between benign and malignant cells, the difference is so far from being clearly defined or consistent that we doubt whether the method can be usefully applied as a diagnostic test of malignancy. The procedure, which requires expensive equipment, is difficult and time-consuming and gives less histological information than routine staining methods. Certainly we have always been able to place more reliance on conventional staining of frozen sections by brilliant cresyl blue for orientation of sections and diagnosis.

It has been shown in the electrophoresis experiments that a moderate excess of soluble tissue protein is capable of binding all the conjugated globulin—that is, the affinity is not limited to a particular group of globulin molecules. Presumably some molecules have specially strong affinity because of complementary structure or high net charge, which might explain the reduction of non-specific staining by tissue absorptions in the fluorescent antibody technique. The histological staining seems likely to be due to non-specific combination of the conjugated N.R.G. with soluble constituents of cytoplasm. Furthermore, the histological

study showed that conjugated N.R.G. resembled eosin in its staining distribution; this might be a result of the net negative charge of the globulin molecule at pH 7.1. Such staining may be contrasted with the specific immunological staining of rat liver described in the preceding paper, in which antiserum was prepared against a microsomal-cell fraction and gave bright staining localized mainly in cell membrane and discrete cytoplasmic particles.

Summary

Investigation of the histological staining reaction of a variety of human and animal tumours with fluorescent conjugates of non-immune rabbit globulin (N.R.G.) has shown variable results with a trend towards equal staining of normal tissue and benign tumours, and diminished staining of carcinomas. The staining was never bright, and with the exception of human colon carcinoma, which stained less brightly than the corresponding normal tissue in almost every case, the differential staining was not consistent. The technique cannot be regarded as a useful practical test for the diagnosis of malignancy.

Centrifugation and electrophoresis experiments on homogenized samples of some tumours and corresponding normal tissues showed that the soluble rather than the particulate components are responsible for combining with the conjugated N.R.G. Combination can take place with all the conjugate, which, possibly because of its net negative charge, behaves as a histological stain in much the same way as standard acid dyes. The diffuse non-specific staining produced by conjugated N.R.G. is different in kind and degree from the bright, defined staining obtained with conjugated organ-specific antisera.

This research programme has been supported by a grant from the Scottish Hospital Endowments Research Trust. We are indebted to Professor J. S. Young, Professor W. O. Kermack, and Dr. M. G. McEntegart for helpful advice, and to Mrs. Evelyn Pirie and Miss Lorraine Rennie for technical assistance. We are grateful to many surgical colleagues at Aberdeen Royal Infirmary and Woodend General Hospital for providing surgical specimens.

REFERENCES

- Adjutantis, G. (1954). *Nature (Lond.)*, **173**, 539.
- Chadwick, C. S., and Nairn, R. C. (1960). *Immunology*. In press.
- Coons, A. H., and Kaplan, M. H. (1950). *J. exp. Med.*, **91**, 1.
- Dineen, J. K., and Ada, G. L. (1957). *Nature (Lond.)*, **180**, 1284.
- Hughes, P. E. (1958). *Cancer Res.*, **18**, 426.
- , Louis, C. J., Dineen, J. K., and Spector, W. G. (1957). *Nature (Lond.)*, **180**, 289.
- King, E. S. J., Hughes, P. E., and Louis, C. J. (1958). *Brit. J. Cancer*, **12**, 5.
- Nairn, R. C., Richmond, H. G., McEntegart, M. G., and Fothergill, J. E. (1960). *Brit. med. J.*, **2**, 1335.
- Weiler, E. (1956). *Z. Naturf.*, **11b**, 31.

The London County Council Health Committee report that there were 177 confirmed cases of polio in London last year, and that more than 80% of those who contracted the disease had not been vaccinated. It is calculated that the rate of attack by paralytic polio in 1959 was seven times greater amongst children who had not been vaccinated than among children who had. In the last six months of 1958 the rate of attack had been five times greater. The Committee consider these figures to be most encouraging, and suggest that the improvement in 1959 may be linked with the fact that by the end of that year slightly over one-half of the children who had been vaccinated had received a third injection of vaccine. (L.C.C. Council Meeting, October 25.)