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The Membrane Antigens of Human Colorectal Cancer Cells: Demonstration with Monoclonal Antibodies of Heterogeneity within and between Tumours and of Anomalous Expression of HLA-DR*

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Abstract—*The membrane antigens of fifteen colorectal tumours were studied using a number of monoclonal antibodies and the immunoperoxidase technique on frozen sections. With this approach we could easily demonstrate differences in the membrane structure of the cancer cells within the tumour mass of given patients and also readily demonstrate differences between tumours that were indistinguishable by histological and other standard criteria. An unexpected finding was the patchy expression of HLA-DR antigens on cancer cells, in spite of the absence of HLA-DR on normal colorectal epithelium. These findings have interesting theoretical and clinical implications.*

INTRODUCTION

BIOLOGICAL behaviour, as measured by parameters such as rapidity of growth, propensity for metastasis and response to particular therapies, varies considerably between tumours of a given type [1]. Moreover, within the tumour of a particular patient, it has often been suggested that a substantial heterogeneity exists and that, for example, metastatic lesions arise from a subgroup of cells from the total tumour mass [2]. What has been lacking is the ability to distinguish either tumours from different patients or the malignant cells within the tumour of a given patient in a manner that might be meaningful from the point of view of its biological behaviour. As the external cell membrane of tumours and all other cells dictates to a very large degree the nature of their interactions with other cells and with their environment in general, it is differences in the make-up of this membrane that will very likely be a vital factor in determining tumour behaviour. For this reason we have been studying the expression on malignant cells of membrane differentiation antigens normally found on the epithelial cells of origin.

In a recently completed study [3] we demonstrated that breast tumours could be subdivided according to whether or not they expressed two membrane antigens found on normal breast epithelial cells. One of those antigens [4], a glycoprotein with a molecular weight of approximately 100,000 daltons, detected by the monoclonal antibody F10-44-2, is also expressed on colorectal epithelial cells (but only on those in the basal half of the crypts of Lieberkühn) and we therefore studied its expression on 15 colorectal tumours. In addition, we examined the tumours for the Mam-3 antigen [5] (found on most colorectal epithelial cells but usually not those at the base of the crypts of Lieberkühn), Thy-1 [6], HLA-DR [7] and HLA-ABC [8]. With these antibodies we could readily distinguish colorectal cancer cells not only between different patients but also within the tumour mass of a given patient. Given the importance of membrane molecules in the interactions of cells with one another and with their environment in general, the importance of HLA-DR and HLA-ABC antigens in immunoregulation and the increas-

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ing emphasis being given to membrane molecules as potential targets for various approaches to cancer therapy, these findings have interesting theoretical and potentially important practical implications. A preliminary brief communication on the anomalous expression of HLA-DR antigens has been submitted [9].

MATERIALS AND METHODS

Monoclonal antibodies

All except two of the monoclonal antibodies were raised in this department and were used as immune ascites partially purified by ion-exchange chromatography [10]. All have previously been described in detail. The F10-44-2 monoclonal antibody [4] is directed at a 100,000 molecular weight glycoprotein originally described on brain and leucocytes and since demonstrated on breast [3] and colonic epithelium, and on a few other tissues (Daar and Fabre, in preparation). The NFK-1 monoclonal antibody [7] is directed at a 'monomorphic' determinant of HLA-DR. It has been fully characterised, but briefly, the antibody precipitates a molecule which has the 2 chain structure typical of HLA-DR antigens and which gives a pattern typical of HLA-DR antigens on 2-dimensional gels. The F15-42-1 monoclonal antibody [6] is directed at the human homologue of Thy-1 [11], a glycoprotein of molecular weight of approximately 25,000 daltons. This antibody was included to check for the anomalous expression of membrane antigens. The F10-89-4 antibody [10] is directed at the leucocyte common antigen, a leucocyte-specific glycoprotein of molecular weight of approximately 200,000 daltons, and it was used to positively identify leucocytic infiltrates. The F3-20-7 antibody [12] is directed at the canine homologue of Thy-1 [11] and was used as a control as it does not react with human tissues. The GD-5 antibody [8] is directed at a monomorphic determinant of HLA-ABC antigens. The Mam-3 antibody was raised against breast epithelial antigens and was a kind gift of Dr. J. Hilgers [5].

Tissues for immunoperoxidase staining

These were collected in the 4 months preceding the study and came from surgical specimens for removal of the tumour. All of the tumours examined were primaries. In some instances pieces of normal colon and rectum were also taken at sites distant from the tumours. They were frozen in liquid nitrogen within 1 hr of removal from the patient, and stored in liquid nitrogen or at −80°C until sectioned.

Immunoperoxidase staining

Cryostat sections of 8 μm were cut, freeze-dried overnight and then fixed for 10 min in acetone at room temperature. Sections were incubated for 30 min at room temperature with saturating concentrations [13] of monoclonal antibody, washed and then incubated for a further 30 min with a 1-in-20 dilution of sheep anti-mouse immunoglobulin serum (kindly provided by Dr. D. Mason, Department of Haematology, John Radcliffe Hospital, Oxford) in 5% normal AB human serum. After washing, peroxidase–anti-peroxidase (PAP) complexes produced by overnight incubation at 4°C of a 1-in-400 dilution of mouse monoclonal antibody to horse-radish peroxidase [14] (kindly provided by Dr. D. Mason) with 10 μg/ml of horse-radish peroxidase (Sigma Chemical Company, London) were added. After 30 min at room temperature the slides were washed and developed with diaminobenzidene and hydrogen peroxide using standard techniques. Sections were lightly counter-stained with haematoxylin.

RESULTS

Normal colon and rectum

As is illustrated in Fig. 1b, the epithelium is negative for HLA-DR antigens, as has been demonstrated also by other groups [15]. The HLA-DR positive structures in the interstitial connective tissue probably represent capillaries [16], interstitial dendritic cells [17] and possibly other cells. The F10-44-2 antigen (Fig. 1c) is found only on the lower half of the crypts of Lieberkühn. Interestingly, Mam-3 (Fig. 1d) shows the converse pattern. Mam-3-positive epithelial cells are found only on the luminal part of the crypt, the basal one-quarter or so being Mam-3-negative. The epithelial cells appear to lose F10-44-2 and acquire Mam-3 as they migrate outwards, an important point to note in relation to the presence or absence of these antigens on some tumours. The F10-44-2-positive cells seen in the connective tissues in Fig. 1c are almost certainly leucocytes [4]. The results with Thy-1 are given in Fig. 1e and show a completely negative epithelium with some positive staining of the connective tissues. HLA-ABC antigens (Fig. 1f) were found strongly expressed both on the epithelial cells and in the connective tissues.

Carcinoma of the colon and rectum

Figure 2b shows a tumour that was positive for the F10-44-2 antigen. All positive tumours were uniformly positive, with no pockets of negative cells, although a little variation in intensity of staining was seen in different regions. The immunoperoxidase technique makes this comment possible and is a distinct advantage over the

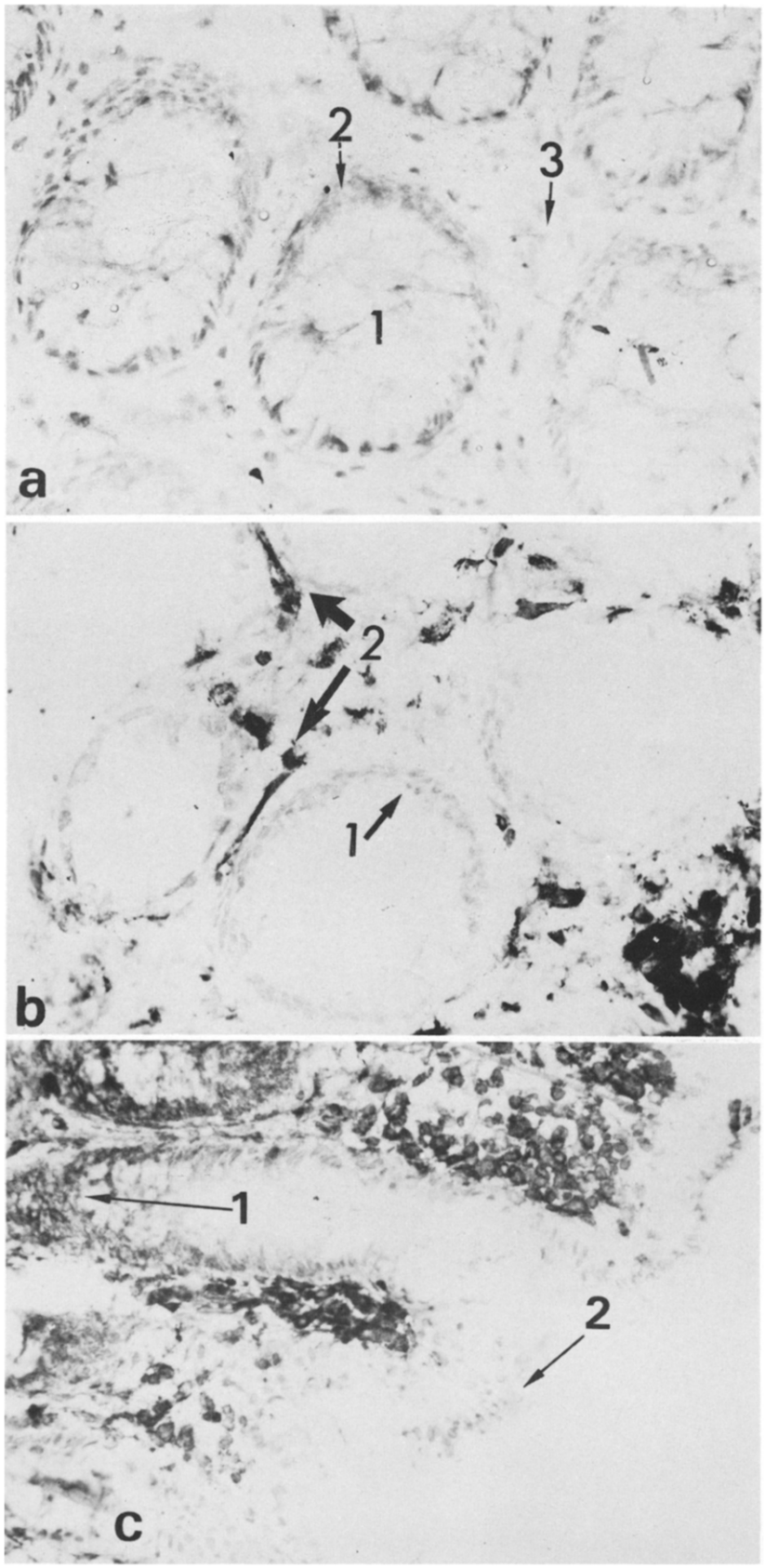

Fig. 1.

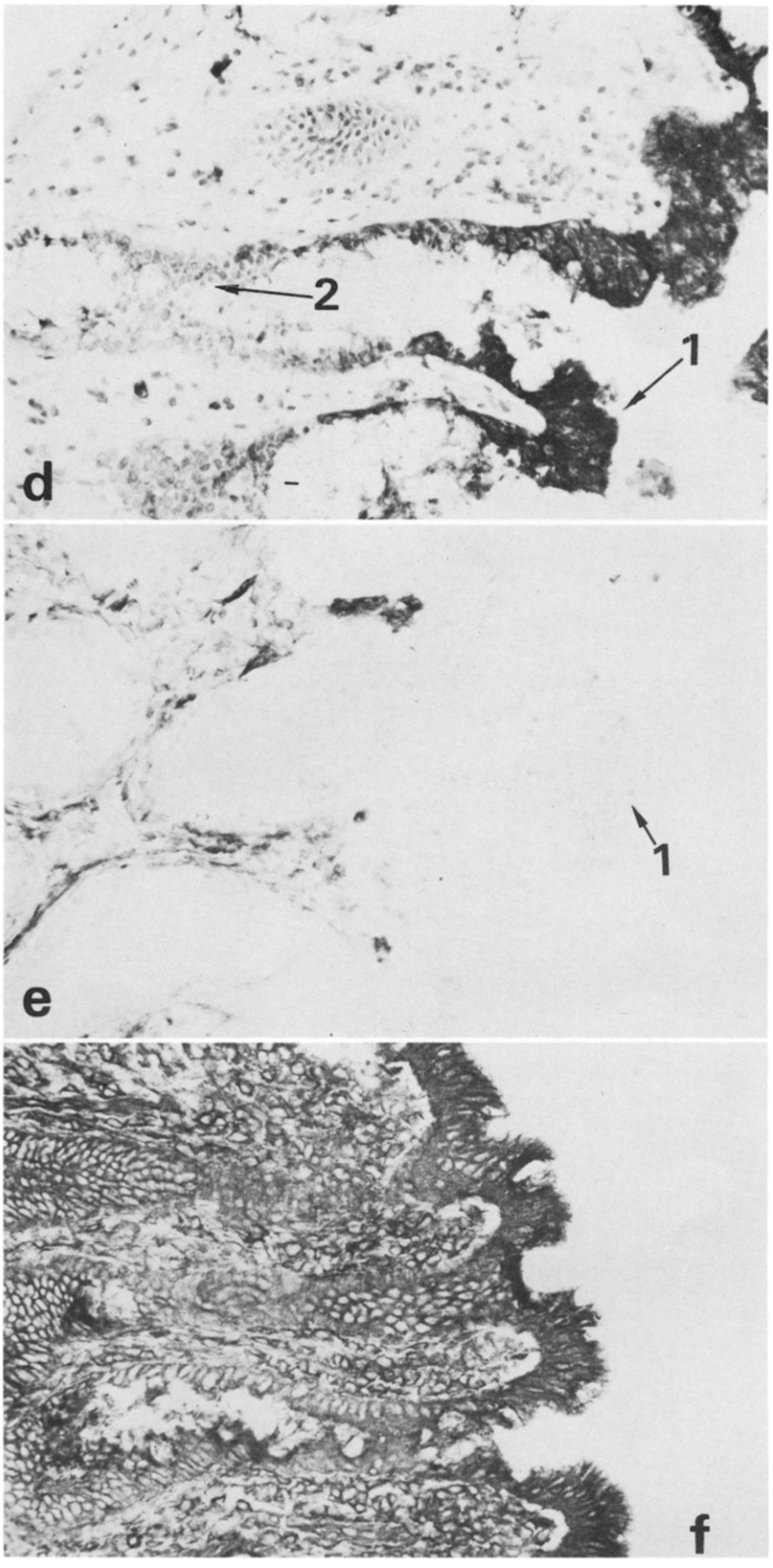

Fig. 1. Immunoperoxidase staining of normal colon. (a) Control antibody: 1, *crypt;* 2, *colonic epithelium;* 3, *interstitial connective tissue. (b) Anti-HLA-DR antibody:* 1, *negative epithelium;* 2, *HLA-DR positive structures in the interstitium. (c)* F10-44-2 *antibody:* 1, *positive epithelium in basal half of crypt;* 2, *negative epithelium in upper half of crypt and surface of colon. (d) Mam-3 antibody:* 1, *positive epithelium;* 2, *negative epithelium. (e) anti-Thy-*1 *antibody:* 1, *negative epithelium. (f) anti-HLA-ABC antibody. (a) and (b)* × 360; *(c)–(f)* × 225.

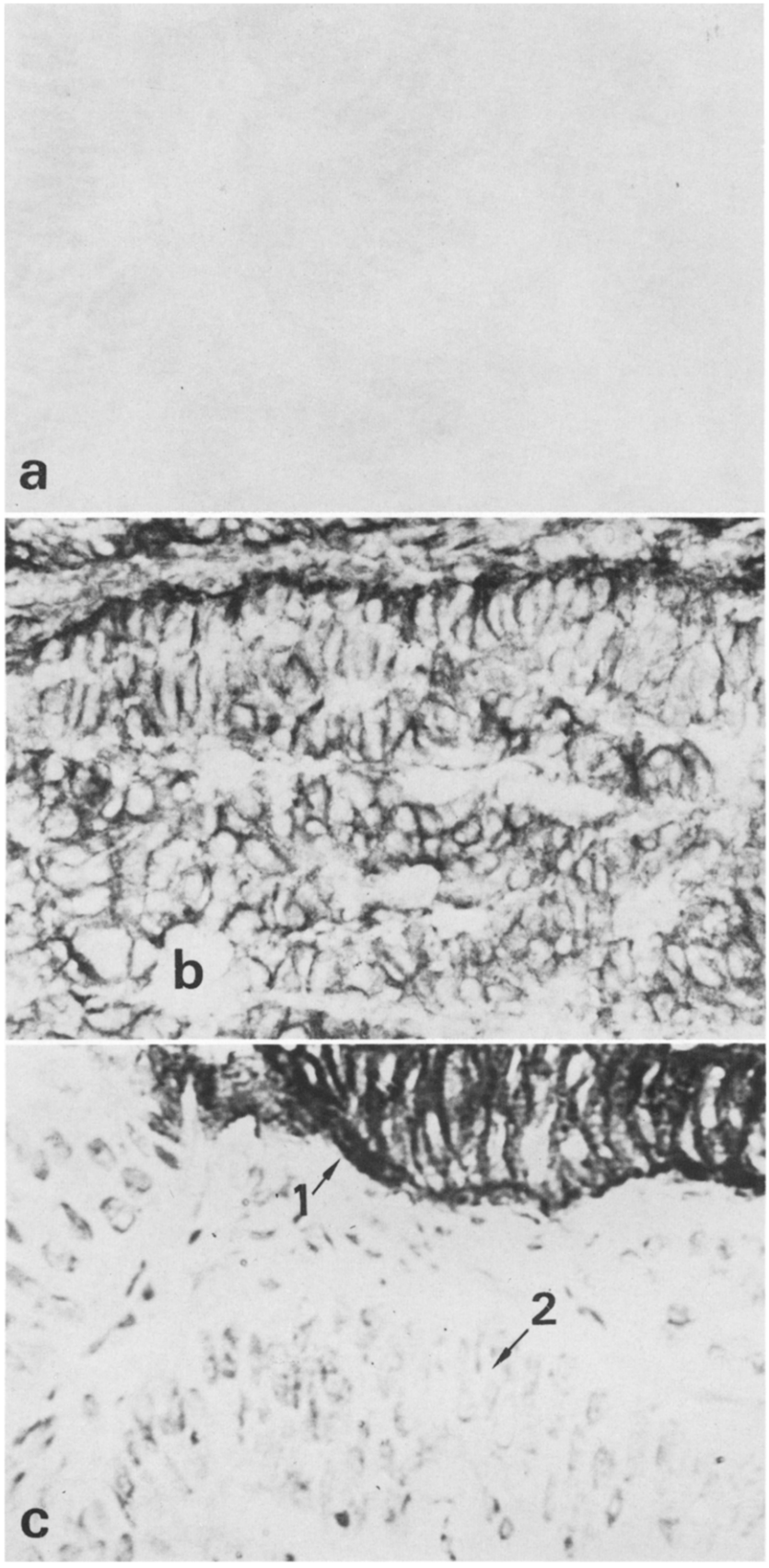

Fig. 2.

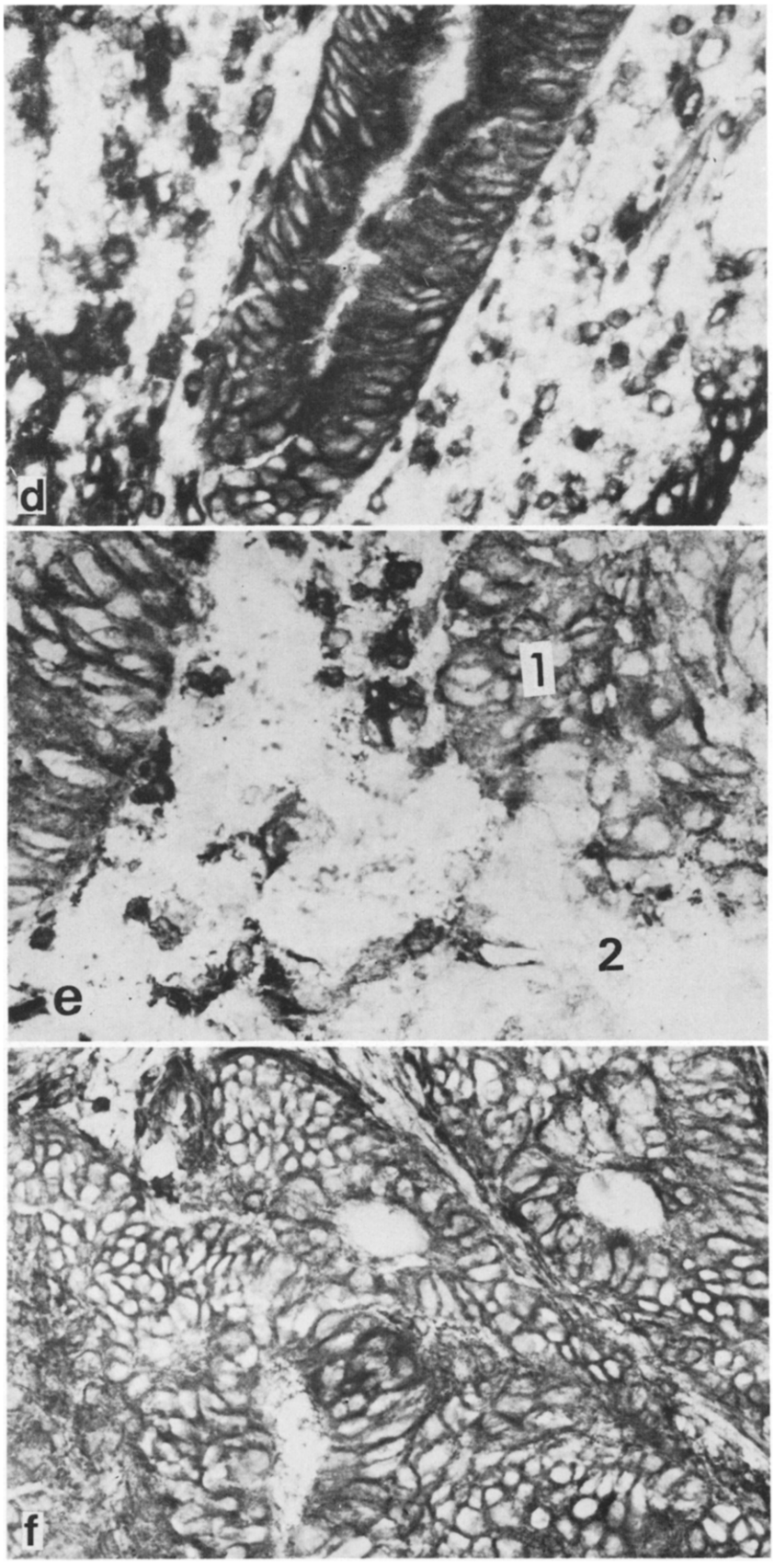

Fig. 2 (contd).

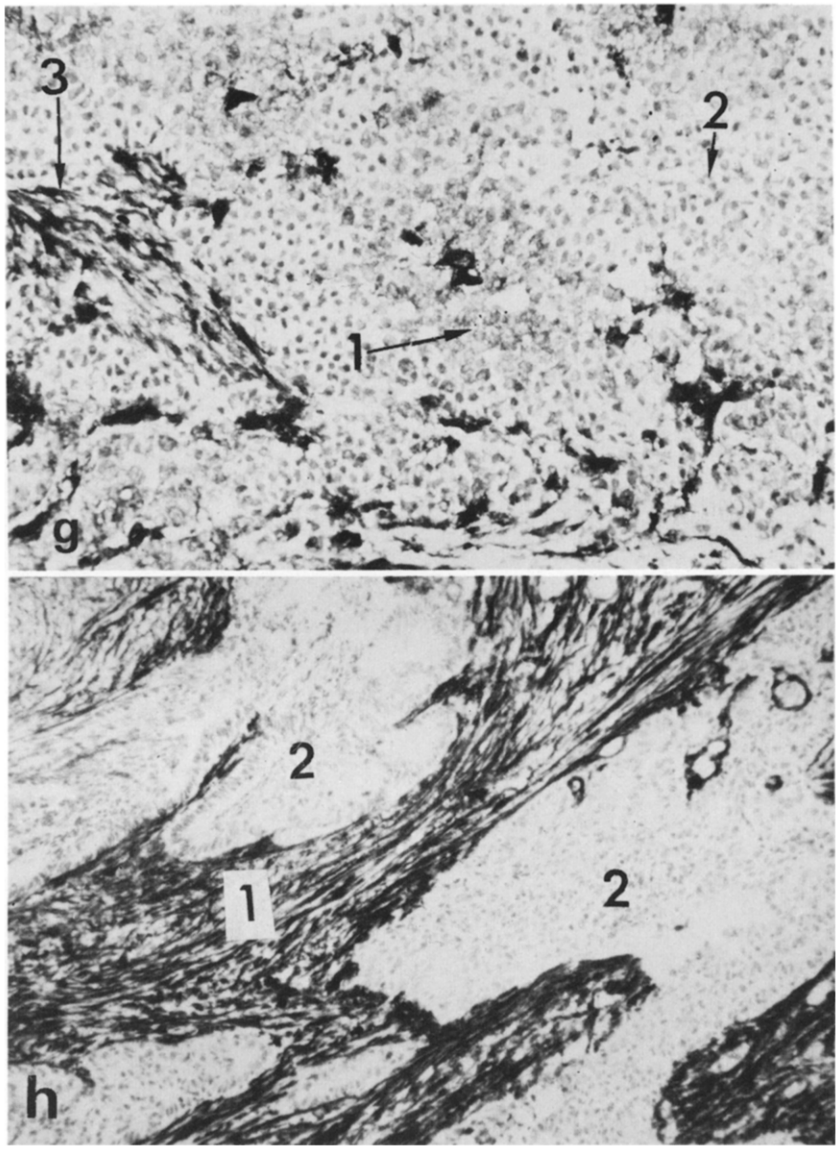

Fig. 2. Immunoperoxidase staining of colorectal cancers. (a) Control antibody, patient D.H. (b) F10-44-2 antibody, patient A.T. (c) Mam-3 antibody, patient A.T.: 1, Mam-3-positive malignant cells; 2, Mam-3-negative malignant cells. (d) Anti-HLA-DR antibody, patient A.J.: a crypt with HLA-DR positive epithelium has been cut in longitudinal section. (e) Anti HLA-DR antibody, patient D.H.: 1, HLA-DR-positive malignant cells; 2, HLA-DR-negative malignant cells. (f) Anti-HLA-ABC antibody, patient D.H. (g) Anti-HLA-ABC antibody, patient U.L.-Y.; 1, HLA-ABC-positive malignant cells; 2, HLA-ABC-negative malignant cells; 3, connective tissue. (h) Anti-Thy-1 antibody: 1, strongly positive interstitial connective tissue; 2, negative malignant cells. The patient code refers to that given in Table 1. (a)–(f), × 360; (g) and (h) × 225.

use of immunofluorescence for this sort of work, since pockets of negative cells could easily go unnoticed with fluorescence. Conversely, all negative tumours were completely negative. The patchy positive areas seen in some breast tumours with the F10-44-22 antibody and immunofluorescence [3] were not observed in this study with colorectal tumours, but as immunoperoxidase has been used in the current study a direct comparison is probably unwise.

In contrast to the clear division of the tumours into those uniformly positive or uniformly negative for F10-44-2, the Mam-3 antigen gave qualitatively different results. All tumours had Mam-3-positive malignant cells, but this was invariably only a proportion of the cells, ranging usually from 10 to 95% of the tumour mass. Figure 2c shows the very clear distinction of the cancer cells into Mam-3-positive and Mam-3-negative. Occasionally the tumour mass was in 2 distinct areas, one positive and the other negative, but more usually the picture was one of clusters of positive or negative cells.

The monoclonal antibodies to HLA-DR were included in the study mainly to study the cells in the interstitial connective tissues (Fig. 1b) as the normal colonic epithelium is negative for these antigens (Fig. 1b and ref. [15]). Paradoxically, however, about half of the tumours expressed HLA-DR antigens.

Two patterns of HLA-DR positivity were seen in the tumour specimens. Figure 2e shows a tumour where, amongst sheets of relatively undifferentiated cells, pockets of strongly HLA-DR-positive cells were seen. These were clearly malignant cells on morphological grounds. Nevertheless, to positively exclude the possibility that these Ia-positive cells might be leucocytes, sequential sections were stained for the leucocyte common antigen [10] and only occasional, isolated cells were found to stain. The second pattern of staining is illustrated in Fig. 2d. Here, what appears to be a relatively well-differentiated crypt, but with intensely HLA-DR-positive epithelium, is seen in longitudinal section. It is not clear whether this represents malignant cells attempting to differentiate into normal (but HLA-DR-positive) epithelial crypts or else the induction of HLA-DR antigens in a normal crypt in close contact with the tumour. Normal bowel mucosa amongst the tumour mass was not always included, so the frequency with which the pattern seen in Fig. 2d was present cannot be commented upon. The HLA-DR-positive patches usually amounted to about 10% of the tumour area seen. In 2 cases where the tumour expressed HLA-DR antigens, normal large bowel epithelium from the same patients was HLA-DR-negative.

Fourteen of the 15 tumours were uniformly and strongly positive for HLA-ABC antigens (Fig. 2f). The one exception was a very poorly differentiated tumour (patient U.L.-Y.), where about half the tumour mass was negative for HLA-ABC antigen and the other half was only weakly positive (Fig. 2g). This is of interest as a much higher incidence of HLA-ABC-negative tumours was recently reported in a study of breast cancers [18].

None of the 15 tumours expressed Thy-1, as expected from the absence of Thy-1 on normal colorectal epithelial cells, but it is of interest in view of the anomalous expression of HLA-DR antigens. We originally included the anti-Thy-1 antibody to study the cells infiltrating the tumours [3]. However, the infiltration was very sparse and, in any case, the interstitial connective tissue was so strongly positive for Thy-1 that the infiltrating cells could not be distinguished (see Fig. 2h).

The results of the 15 colorectal cancers examined in this study are summarised in Table 1. It can be seen that 10 of the 15 tumours were positive for the F10-44-2 antigen and that the presence or absence of the antigen could not be correlated with the degree of differentiation (as assessed by conventional histopathological criteria) or the site of origin of the tumour, Duke's classification, the presence of HLA-DR or the proportion of Mam-3-positive cells. Seven of the 15 tumours had patches of HLA-DR-positive malignant cells as seen in Fig. 2e, occasionally with, in addition, the pattern seen in Fig. 2d. As for F10-44-2, the presence of HLA-DR-positive malignant cells could not be correlated with any clinical criteria or with the expression of the other membrane antigens studied. There was also no correlation with the degree of leucocytic infiltration, which was, in any case, minimal at best.

The one benign villous adenoma examined was uniformly positive for both the F10-44-2 and Mam-3 antigens, which is worth noting in view of the expression of these antigens on opposite, although overlapping, regions of the crypts of Lieberkühn. The adenoma was also uniformly positive for HLA-ABC antigens and completely negative for the HLA-DR and Thy-1 antigens.

DISCUSSION

Four main findings emerge from this study. Firstly, we demonstrate that human colorectal cancers can be subdivided according to whether or not they express the F10-44-2 antigen, a glycoprotein of molecular weight 100,000 daltons found on normal colorectal epithelium as well as breast epithelium [3] and a few other tissues [4]. Normally the loss of a membrane antigen by a

Table 1

Patient	Histological differentiation	Site	Duke's classification	Liver secondaries	Staining* with anti: F10.44.2	Mam-3	Thy-1	HLA-DR	HLA-ABC
J.W.	Good	Rectum	C	Absent	-	90%	—	—	++
M.B.	Moderate	Rectum	C	Absent	+	50%	—	10%	++
H.B.	Good	Sigmoid	B	Not recorded	+	95%	—	—	++
T.L.	Poor	Caecum	B	Absent	+	90%	—	—	++
D.H.	Good	Rectum	B	Not recorded	+	95%	—	10%	++
A.J.	Moderate	Rectum	B	Absent	-	10%	—	10%	++
A.T.	Moderate	Sigmoid	B	Absent	+	25%	—	—	++
C.W.	Good	Rectum	B	Absent	+	50%	—	10%	++
C.S.	Moderate	Sigmoid	B	Absent	+	50%	—	—	++
B.C.	Moderate	Splenic flx	C	Present	+	10%	—	10%	++
C.H.	Moderate	Hepatic flx	B	Not recorded	+	ND	—	10%	++
A.M.	Poor	Rectum	C	Present	-	90%	—	—	++
L.P.	Moderate	Rectum	B	Not recorded	+	90%	—	—	++
A.K.	Good	Rectum	C	Absent	-	95%	—	10%	++
U.L.-Y.	Very poor	Splenic flx	C	Absent	-	50%	—	—	50%
				Positive:	10/15	14/14	0/15	7/15	15/15

*++ Indicates uniform staining of high intensity; + indicates uniform staining of moderate intensity; - indicates no staining at all of the tumour cells. A percentage figure indicates heterogeneity in the tumour, and gives the approximate proportion of the tumour mass that was positive. ND indicates 'not determined'.

tumour cell would be good biochemical evidence for loss of differentiation, but the interpretation of the loss of F10-44-2 antigen by colorectal cancer cells is complicated by the fact that the F10-44-2 antigen is found on only the lower half of normal crypts of Lieberkühn. Clearly, as the cells age or mature in their movement up the crypt they normally lose the F10-44-2 antigen. Loss of the antigen by the 5 tumours shown in Table 1 can therefore be taken as evidence either for loss or attainment of maturity, and it is not possible at the moment to distinguish between these 2 divergent possibilities. Whatever the explanation, however, the presence or absence of the F10-44-2 antigen is likely to be a subtle test of differentiation (in one direction or the other) and to have functional implications, e.g. in metabolism or cell-to-cell interactions for the tumour. Therefore, as previously discussed in detail for breast tumours [3], our finding has scope for clinical exploitation, and a long-term clinical trial is currently underway to see if any correlation can be made between the presence or absence of the F10-44-2 antigen on the tumour and its clinical behaviour in terms of patient survival, propensity for metastasis and response to therapy.

The second and unexpected finding was the anomalous and patchy expression of HLA-DR antigens on about half of the colorectal cancers we examined. This finding can be exploited in clinical trials in much the same way as has just been described for the F10-44-2 antigen because the presence of HLA-DR-positive malignant cells might be of functional importance to the tumour as a whole. For example, given that HLA-DR antigens are important in antigen presentation to helper T lymphocytes [19], the presence of HLA-DR-positive cells might augment the immunogenicity of any tumour-specific antigens and thereby be of positive benefit in controlling tumour growth.

The third finding was the invariably patchy expression of the Mam-3 antigens by all the tumours, a pattern different to that seen with either F10-44-2 (where all tumours were either uniformly positive or uniformly negative) or HLA-DR (where half the tumours were completely negative). The fact that a subpopulation of tumour cells can be simply and readily distinguished from the tumour population as a whole raises interesting possibilities in terms of studying any unusual characteristics of these cells. For example, what is the HLA-DR and Mam-3 status of secondary tumours? Can one detect preferential resistance or susceptibility to chemotherapeutic or other agents of the HLA-DR- or Mam-3-positive malignant cells by studying the tumours before and after therapy? Will longitudinal studies show preferential expansion or loss of HLA-DR- or Mam-3-positive cells? All of these points, and the effect of F10-44-2 and/or HLA-DR antigen on clinical outcome, are questions that are readily amenable to investigation by careful clinical and laboratory studies.

The fourth finding was the absence of HLA-ABC antigens on approximately half of the tumour cells in patient U.L.-Y., whose tumour had been classified as very poorly differentiated. It has been suggested that the absence of HLA-ABC antigens might render cancer cells no longer susceptible to the attack of cytotoxic T cells [18] because of the phenomenon of MHC restriction, which requires the presence of self HLA-ABC antigens (in addition to the target antigen) on the targets of cytotoxic T cells [20]. The whole question of the role of the immune system and of tumour antigens in the control of tumour growth is entirely an open one. However, if MHC-restricted cytotoxic T cells and tumour antigens are indeed important controlling influences, one could speculate that the *mechanism* whereby the presence of poorly differentiated anaplastic cells confers a poor prognosis is specifically and particularly the loss of HLA-ABC antigens and their consequent escape from the immune control.

There are perhaps two further points worth discussing. The first is the possible significance of the HLA-DR-positive crypt seen in Fig. 2d. This probably represents the induction of HLA-DR antigens as a result of local host responses to the tumour, particularly in view of the recent finding that systemic graft-vs-host disease induces the expression of Ia antigens in the skin [21, 22] and colonic epithelium [22] of the rat. However, it is also possible, although we think it unlikely, that induction of HLA-DR antigens in the colonic epithelium predates the development of the tumour and might therefore represent a localised premalignant change.

The second point is that our studies forcefully illustrate one of the major problems associated with the various approaches using tumour cell antigens as targets for tumour therapy [23], particularly since the advent of monoclonal antibodies, and that is tumour cell heterogeneity.

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