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Phenotypic expression of histocompatibility antigens in human primary tumours and metastases

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HLA class I and II expression was studied on 244 (177 primary and 67 metastatic) solid human tumours of different origin. Alkaline immunophosphatase (APAAP) and immunoperoxidase were used on cryostatic sections to stain MHC antigens. Monomorphic MoAbs were used against class I heavy chain, β2-microglobulin, DR, DQ and DP molecules.

Class I expression was homogeneous on colon, melanoma and epidermoidal primitive tumours. Loss of HLA class I antigens was more frequent on basal cell carcinomas and sarcomas and was related to tumour differentiation on larynx carcinoma. Class I expression was heterogeneous on breast, larynx and stomach primitive neoplasias. Class I negative tumours were more frequent on metastatic than on primitive melanomas. Divergence of class I between primary tumours and autologous metastases was observed on melanomas, larynx and colorectal carcinomas.

Class II expression was heterogeneous on all tumours and in a large number of cases was associated with high intensity of leukocytic infiltrate. HLA-DR expression was higher than HLA-DP and HLA-DQ (DR > DP > DQ) and was related to tumour progression. Four human tumour cell lines were modulated with recombinant interferon-γ for HLA class I and II antigens. Different HLA profiles were obtained: increased class I and II expression, increased class II or a low response.

Finally, class I genes from 22 tumours were compared with autologous normal cells by Southern blot analysis: 12 tumours were class I positive and 10 negative. No clear differences in RFLP were observed that could be associated with class I rearrangement. The results are discussed in relation to the role that histocompatibility antigens may play in tumour progression and invasiveness.

Introduction

The products of class I histocompatibility antigens are 45 kd polymorphic cell surface glycoproteins, present on most somatic cells in association with β2-microglobulin [29]. These antigens function as targets directing the attack of cytolytic T lymphocytes (CTLs) against virally infected or neoplastic cells [39].

The class II histocompatibility antigens are composed of two polypeptides, the α and β chains, weighing about 34 and 28 kd, respectively, and comprise the gene products of the DR, DP and DQ loci located in the HLA-D region [32]. These molecules act as restriction elements for T helper cells [14].

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Much evidence suggests that alterations in expression may play a crucial role in oncogenesis and metastasis [18]. In several tumour systems the absence of class I histocompatibility antigen expression has been correlated to greater tumour aggressiveness [16, 17]. Furthermore, the participation of these molecules in tumoral rejection has been confirmed in gene transfection experiments [21]. Other reports suggest, however, that the increase of a particular class I molecule may be related to more invasiveness and metastasis [12].

This paper describes a study of class I and II histocompatibility antigen expression in 244 surgically removed tumours (177 primary tumours and 67 metastases) of different origin (colon, larynx, breast, melanoma, stomach) and Southern blot analysis of class I genes.

Materials and methods

Tumour specimens

Most of the tissues were obtained from patients being seen in the Department of General Surgery. None of the patients had received radiotherapy and/or chemotherapy prior to surgery. Histopathologic diagnosis was confirmed in paraffin sections. All tissues were snap-frozen in liquid nitrogen cooled with isopentane after coating with OCT within 1–2 h of removal and were stored in liquid nitrogen until sectioning for study.

Cryostatic sections 5 μm thick were cut and allowed to dry at room temperature for 4–18 h, after which they were fixed for 10 min in acetone, wrapped in aluminium foil and stored at −4°C until stained.

Alkaline immunophosphatase staining. Sections were incubated with the first antibody in a humidified chamber at room temperature for 45 min. After washing with 0·05 M Tris-buffered saline (pH 7·6) (TBS). Sections were first incubated for 30 min with rabbit antimouse immunoglobulin (1 : 20) (DAKO), washed with TBS and reincubated for 30 min with APAAP complex (DAKO). After washing with TBS, chromogen was added (0·2 mg/ml naphthol AS-MX phosphatase and 1 mg/ml Fast Red TR salt) (Sigma). A final washing with TBS was followed by counterstaining with haematoxylin and mounting with Apathy's medium (Raymond Lamb, London).

Immunoperoxidase staining. Tissue sections were acetone fixed for 10 min at 20°C, washed in PBS and incubated with 25 μl of the particular antibody for 30 min at 20°C. After washing in PBS, a second layer consisting of horseradish peroxidase-conjugated rabbit antimouse immunoglobulin (1 : 40) (DAKO) containing human serum AB (1 : 100) was applied for 30 min at 20°C. After washing in PBS followed by TBS, the peroxidase label was visualized by incubation with 0·06 per cent DAB (Sigma) in 0·12 per cent H_2O_2 in Tris–HCl (pH 7·6) for 8 min. Sections were counterstained with Harris's haematoxylin (Sigma) and mounted in DPX mountant (BDH).

Samples were considered positive (+) when more than 10 per cent of cells were clearly stained.

Monoclonal antibodies

The following monoclonal antibodies against MHC gene membrane antigens were used: W6/32 against HLA ABC [3], kindly provided by Dr W. F. Bodmer; GRH1 against β2-microglobulin, produced in our laboratory [24]; GRB1 against DR, produced in our laboratory [9]; B7/21 against DP [37]; and Tü22 against DQ

[38], kindly provided by Dr P. Wernet. In addition, GRT2, a monoclonal antibody produced in our laboratory and directed against the T200 molecule (CD45), was used to measure the leukocytic infiltrate in tumours [24]. A 1 : 100 final dilution ascitis was used in all cases.

In vitro *treatment with interferon-γ (IFN-γ)*

Tumour cell lines were obtained by mechanical disruption and collagenase and trypsin treatment. Cells were cultured in RPMI 1640 supplemented with glutamine and 20 per cent (v/v) foetal bovine serum. Human recombinant IFN-γ (Böhringer, Ingelheim, F.R.G.) had a titre of $4{\cdot}6 \times 10^7$ units/ml and a specific activity of 2×10^7 units/mg of protein. Tumour cell cultures were grown in 25 ml culture flasks with 50 U/ml of IFN-γ. We therefore cannot completely exclude the possibility that the cells growing in culture were not tumour cells; there are indications in some lines (Co3, G1 and G2) of lack of contact inhibition and absence of haematopoietic markers (CD45, CD3, CD20). With regard to the melanoma line M4 the presence of premelanosomes observed by electron microscopy confirmed the origin of the cells. Induction of HLA antigens was analysed at 24, 48, 72 and 96 h by a radiobinding technique. The MoAbs were added in triplicate tubes containing 2×10^5 cells at a 1 : 50 final dilution, and incubated for 1 h at 4°C. The cells were washed with PBS–BSA three times; 50 μl of PBS containing 5×10^4 cpm of $[^{125}I]F(ab')_2$ goat anti-mouse Ig (specific activity 15 μCi/μg) (Amersham) were added to each tube. After 1 h at 4°C the cells were washed four times. The cell-bound radioactivity was measured in a gamma counter.

Southern blot analysis

DNA was isolated from lymphocytes or autologous normal mucosa and tumours by phenol–chloroform extraction [7]. DNA samples (10 μg) were digested with at least 100 units of EcoR I, Hind III, PvuII (Amersham), electrophoresed in 0·8 per cent agarose gels and transferred to nitrocellulose membranes [34]. The membranes were hybridized with a cDNA probe of 1·4 kb, containing most of the coding sequences of HLA B7 molecule [33]. The probe was nick translated (^{32}P-labelled) to approximately 10^8 cpm/μg. Hybridization was performed in 50 per cent formamide hybridization buffer at 42°C for 36 h. The filters were washed at 65°C with 3 × SSC and 0·1 × SSC supplement with 0·1 per cent SDS and autoradiographed at −70°C.

Results

Expression of class I and II antigens in primary tumours

Tables 1 and 2 present the results obtained with monoclonal antibodies against HLA-ABC, β_2-microglobulin and DR, DP and DQ molecules in a total of 177 cases. In most cases the staining patterns were membranous and cytoplasmic. No differences were seen between anti-heavy chain (W6/32) and anti β_2-microglobulin (GRH1) MoAbs. In the case of class II antigens, staining patterns similar to those just described were observed, whereas with these molecules notable differences were revealed both in the frequency of staining with DR and in the variable intensity of expression (DR > DP > DQ).

Further differences were noted in the numbers of cells stained in each tumour. Whereas colon, melanoma and epidermoidal tumours were markedly homogeneous

Table 1. Class I and II expression in primary tumours.

	Class I(+/total)[hi]	Class II(+/total)[j]		
Tumours	β2-m and H chain	DR	DP	DQ
Colon[a]	29/34	18/34	5/34	3/34
Stomach[b]	8/10	2/10	2/10	2/10
Breast[c]	33/39	15/39	N.T.	N.T.
Larynx[d]	18/24	2/24	0/24	0/24
Melanoma	17/19	6/19	N.T.	N.T.
Renal[e]	12/13	7/13	2/7	3/7
Sarcoma[f]	0/4	1/4	1/4	1/4
Epidermoidal[g]	7/8	5/8	N.T.	N.T.
Basal cell[g]	17/26	2/26	N.T.	N.T.

[a] 30 adenocarcinomas and 4 mucoid carcinomas.
[b] Adenocarcinomas.
[c] Ductal infiltrating carcinomas.
[d] Squamous cell carcinomas.
[e] 11 renal carcinomas and 2 adenocarcinomas.
[f] 1 rhabdomiosarcoma, 2 malignant histiocitomas and 1 malignant schwannoma.
[g] Skin basal cell and epidermoidal carcinomas.
[h] (+/total) = positive/tested.
[i] Class I antigen staining patterns were generally homogeneous in melanoma, colon and epidermoidal cancer and heterogeneous in stomach, breast, larynx cancer and basal cell carcinoma.
[j] Class II antigen expression was generally heterogeneous.

Table 2. Class I and II antigen expression in metastatic tumours.

	Class I (+/total)	Class II (+/total)		
Tumours	β2-m and H chain	DR	DP	DQ
Colon	13/13	5/13	1/13	0/13
Stomach	11/17	3/17	3/17	3/17
Breast[a]	0/1	0/1	0/1	0/1
Larynx	7/10	0/10	0/10	0/10
Melanoma	12/23	7/23	N.T.	N.T.
Renal[a]	1/1	1/1	1/1	1/1
Rhabdomyosarcoma[a]	0/1	0/1	0/1	0/1
Epidermoidal[a]	1/1	1/1	1/1	1/1

[a] Homogeneous and heterogeneous patterns were similar to those seen in primary tumours.

(figure 1a), breast, larynx and stomach neoplasias were mostly heterogeneous for class I antigens (figure 1b). All four cases of sarcoma in the present study were completely negative. Moreover, the frequency of loss of histocompatibility antigen expression (figures 1c and d) clearly varied among the different kinds of tumours. Colorectal carcinomas and melanomas kept such expression most frequently (86 and 95 per cent respectively) while basal cell carcinomas and sarcomas were the types which most commonly lost expression (34 and 100 per cent, respectively).

Generally, class II antigen expression was fairly heterogeneous and tended to be focal. Only two cases of very well differentiated larynx cancer showed homogeneous expression of DR antigen. However, very limited and localized class II antigen expression was detected in some of the remaining tumours. In a large number of cases class II antigen expression was associated with high intensity of leukocytic infiltrate.

Expression of class I and II antigens in metastatic tumours

A total of 67 cases of metastases were studied, mostly representing infiltration of lymph nodes in the vicinity of the tumour (table 2).

The expression of class I and II antigens was similar in many cases to the picture in primary tumour cells. The staining patterns with anti β_2-microglobulin and anti-heavy chain MoAbs paralleled the results in primary tumours. The intensity of expression DR > DP > DQ also replicated findings described in the previous section.

A remarkable difference with the primary tumour was the greater frequency with which melanoma metastases lost class I antigen expression: 12/23 positive cases as opposed to 17/19 class I positive primary tumours. No such phenomenon was observed with class II antigen expression on melanomas, although differences were seen regarding the location of metastases: 7/15 ganglionar metastases HLA class II positive in comparison with 1/8 cutaneous metastases.

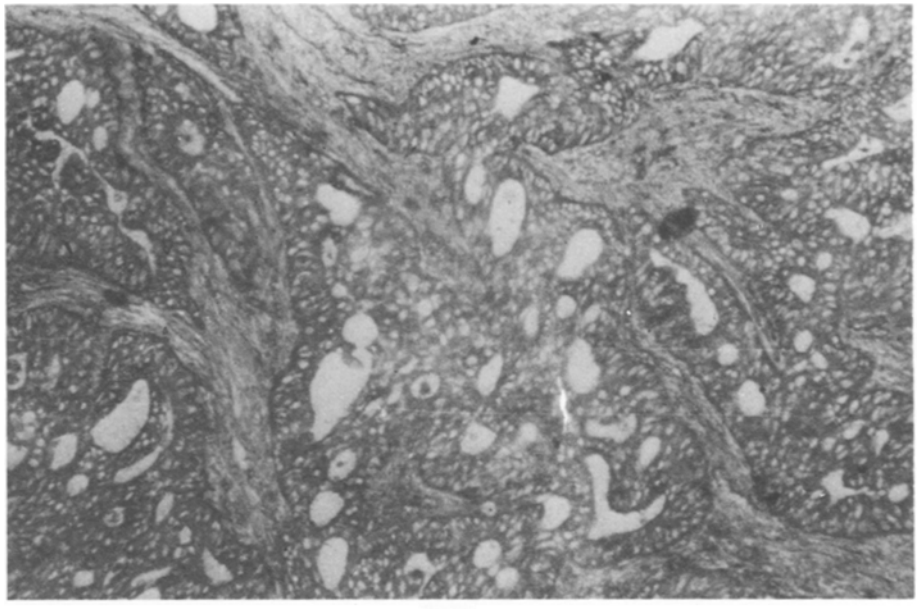
Comparison of class I and II antigen expression in autologous metastasis and primary tumours

Some metastases failed to express class I antigens previously detected on the primary tumours from which they had developed, whereas the opposite situation was found in other cases (table 3). Representative instances of such tumour metastases divergence were seen mostly in melanomas and larynx squamous cell carcinoma. In the remaining cases no such divergence was observed. In 8 out of 13 colon metastases a smaller number of class I positive cells was observed than on autologous primary tumours. Class II antigen expression also yielded well correlated findings between primary tumours and autologous metastases. In two cases of colon cancer the antigen was more intensely expressed either in primary tumour or autologous metastases and one metastatic melanoma lost class II antigen expression.

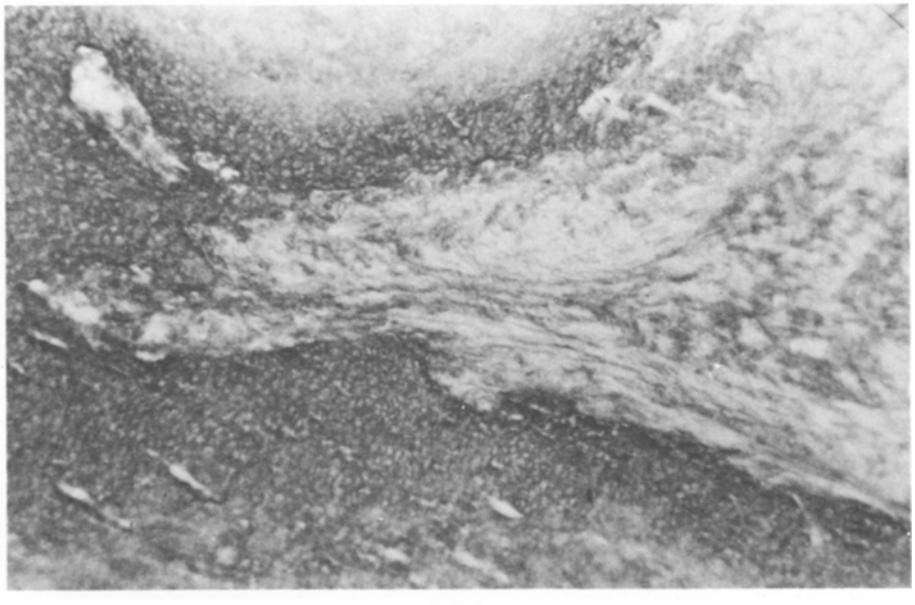
Modulation of class I and II HLA antigens after treatment with interferon-γ.

Four human cell lines, two obtained from gastric carcinomas G1 and G2, one from a colorectal carcinoma (CO3) and one from a melanoma (M4), were analysed for class I and II HLA antigen expression after treatment with recombinant IFN-γ.

Class I expression was increased in the four cell lines to different extents and with different patterns (figure 2). For instance, a gastric cell line (G1) showed a rapid increase in the amount of class I whereas the other (G2) showed a later induction



(*a*)



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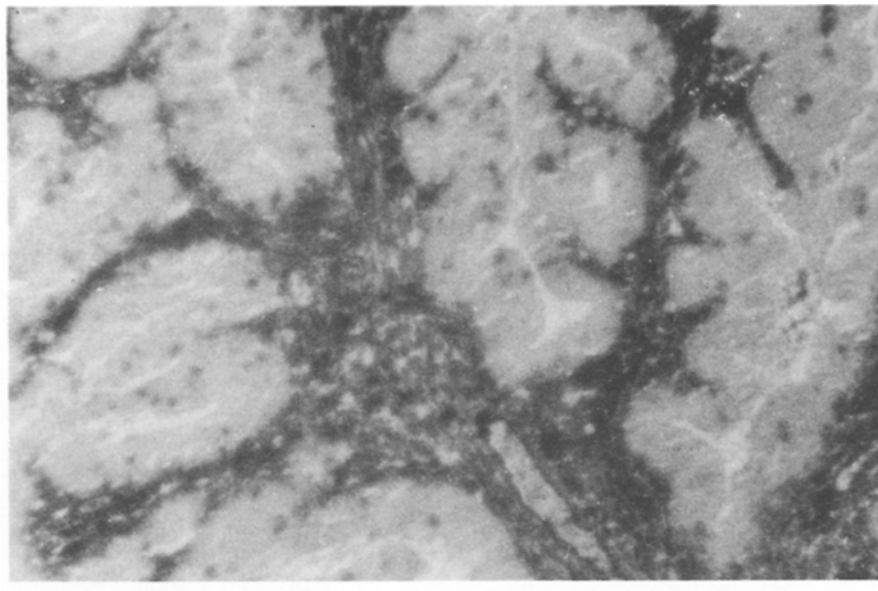

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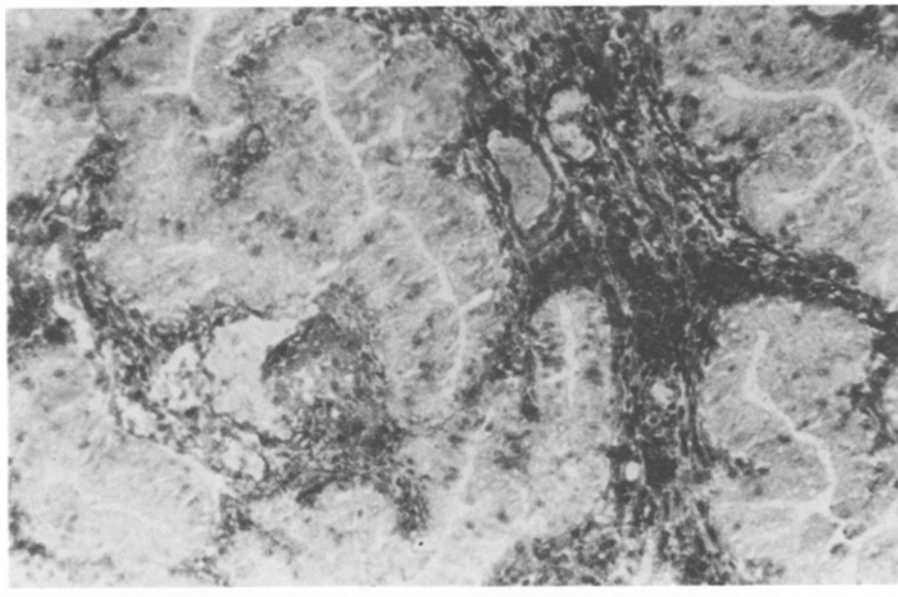

(*d*)

Figure 1. (*a*) Colon adenocarcinoma showing homogeneous positive staining with MoAb W6/32, mainly on cell membrane. Counterstained with haematoxylin (× 400). (*b*) Well differentiated epidermoid carcinoma of the larynx showing heterogeneous class I antigen expression with W6/32. Class I expression was more intensive in actively dividing cells. Both membrane and cytoplasm are positively stained (× 200). (*c*) Colon carcinoma showing negative staining for class I antigen. Red stained interstitial cells are infiltrating lymphocytes. APAAP technique was used. Counterstained with haematoxylin (× 400). (*d*) Colon carcinoma stained for HLA class I and showing strong expression on stroma lymphoid cells but not on the tumour cells. Immunoperoxidase staining and counterstained with haematoxylin (× 200).

after incubation for 3 days. The colorectal cell line (CO3) expressed a basal high level of class I antigen and IFN-γ induced a slight increase. The melanoma cell line (M4) was class I negative and re-expressed these antigens after IFN-γ treatment.

Class II expression was induced on the four tumour lines by IFN-γ. These four lines were class II negative. The effect of IFN-γ on each line was different, as with class I. The highest response was observed on the colorectal line CO3 and the lowest on the gastric line G2. The other gastric line G1 showed a progressive and moderate increase in class II expression. The melanoma line M4 reached a high class II expression after incubation for 48 h and a fall of class II expression was observed at 72 h.

Comparative analysis of class I genes between tumours and autologous normal cells

RFLPs for class I genes were analysed by Southern blot on 22 tumours (10 colorectal carcinomas, 3 melanomas, 7 gastric and 2 laryngeal carcinomas) and autologous normal cells. Twelve tumours were class I positive and ten class I negative. We found no clear differences between tumour and autologous normal mucosa (figures 3a and b).

Table 3. Expression of class I and II antigens in primary tumours and autologous metastases.

		Class I antigens[d]		Class II antigens[d]	
Case	Site[a]	Primary	Metastasis	Primary	Metastasis
Colon	Liver	+	+	−	−
Colon	Peritoneum	+	+	+↑	+
Colon	Liver	+↑	+	−	−
Colon	L. node	+	+	+	+↑
Colon	L. node (2)	+	+	−	−
Colon	L. node (8)	+↑	+	−	−
Stomach	L. node (6)	+	+	−	−
Stomach	L. node (3)	+	+	+	+
Stomach	L. node	−	−	−	−
Larynx	L. node (4)	+	+	−	−
Larynx	L. node (2)[b]	+	−	−	−
Larynx	L. node (2)[b]	−	+	−	−
Larynx	L. node	−	−	−	−
Melanoma	L. node[b]	+	−	−	−
Melanoma	L. node[b]	−	+	+	+
Melanoma	L. node[c]	−	+	+	−
Melanoma	L. node[b]	−	+	−	−
Rhabdomyosarcoma	L. node	−	−	−	−
Breast	L. node	−	−	−	−

[a] (n) = number of cases.
[b] Divergence of class I expression between primary tumours and metastases.
[c] Divergences of class I and II.
[d] ↑, Increased degree of staining; +, >10% of the cells stained.

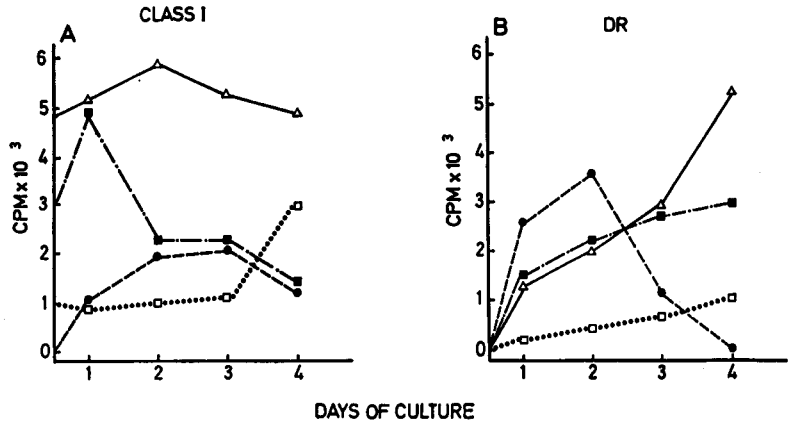

Figure 2. Modulation of HLA antigens by IFN-γ. Radiobinding assays with MoAbs: (*a*) W6/32 for class I; (*b*) GRB1 for HLA-DR. All values are means of triplicate determinations and represent specific binding, cpm $\times 10^{-3}$ per tube $\times$ S.D. Non-specific binding was measured using X63 supernatant, a mouse myeloma protein, and subtracted. (△) A colorectal cell line (CO3); (■) a gastric cell line (G1); (□) a gastric cell line (G2); (●) a melanoma cell line (M4).

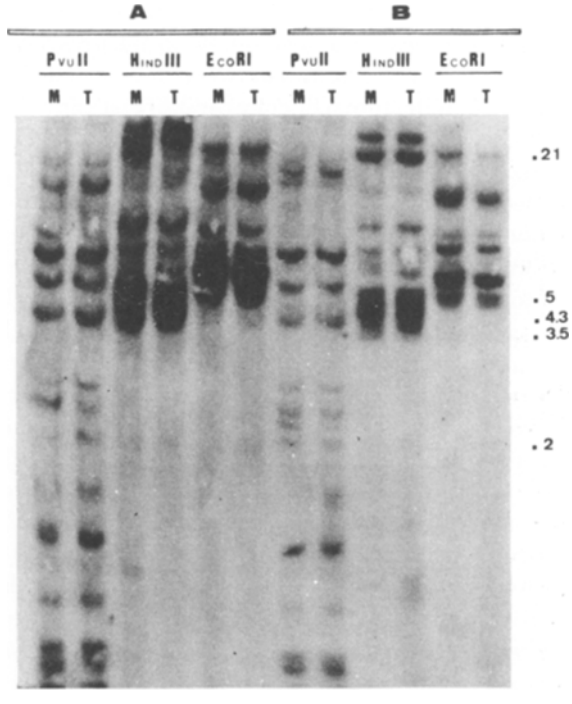

Figure 3. RFLP patterns of tumour cells (T) and autologous normal mucosa (M). DNAs digested with the indicated endonucleases and hybridized with HLA probe (pDP001). (*a*) Class I positive and (*b*) class I negative gastric carcinomas. The intensities of the signals were similar in normal and tumour samples.

Discussion

Three types of change of histocompatibility antigen expression on tumour cells have thus far been described: loss of expression, enhancement and the appearance of new antigens [15, 19].

The loss of class I expression has been correlated with the degree of differentiation, histologic variant or invasiveness of tumours [13, 25, 35]. The loss of the restriction element at the tumour cell surface is one mechanism involved in the escape of tumour cells from cytotoxic T lymphocytic attack as studies of an adenovirus transformed cell line have shown [6]. However the existence of an alternative surveillance mechanism, which operates by detecting the absence of the expected (MHC) rather than the presence of the unexpected (antigens), has been postulated [23, 27].

In this study, most primary tumours revealed marked class I expression, except in the four cases of sarcoma. Class I expression was generally uniform in most melanomas, colorectal and epidermoidal cancer, heterogeneous in breast, stomach and larynx tumours and most cutaneous basal cell carcinomas and completely negative in all four sarcomas (table 1). In both laryngeal cancer and basal cell carcinoma, class I antigen expression was more intensive in actively dividing cells, although these cells lose ABC antigens as they mature. However, although some tumours (colon, breast, stomach) can be correctly said to lose class I antigens which are expressed on normal mucosa [11, 28], this is not the case in displastic nevocellular nevus and primary cutaneous melanoma, which show a marked increase in class I antigen expression. In the course of malignant transformation of melanocytes, expression becomes more intense in association with the presence of T lymphocytic infiltrate [5, 31]. No correlation was found between degree of differentiation and loss of class I antigen expression in stomach, colon and breast cancer. In contrast, 14/18 well differentiated laryngeal carcinomas were found to be HLA class I antigen positive, whereas 5/6 class I negative tumours were poorly differentiated and one was moderately well differentiated.

Lack of expression also has been correlated with class I gene rearrangement or deletion in some transformed cells [30]. In most cases, however, some regulatory mechanism is likely to intervene at the transcriptional level. Class I expression can be induced by several agents such as IFN-γ. Our melanoma line class I negative re-expressed these antigens after incubation with IFN-γ. In the other three lines we observed a variable increase in class I expression. The different profiles of class I induction with IFN-γ detected in the four lines may be due to the different histological origin or to variations of IFN-γ receptor level [36]. The increase in class I expression by IFN-γ may be related to its antitumoral effect because IFN-γ also induces tumour-associated antigen expression and these two factors together are favourable for triggering cytolytic antitumoral T lymphocytes [10].

We have compared class I genes by Southern blot analysis of HLA class I positive and negative tumour cells (melanoma, colon, gastric and laryngeal carcinomas) with peripheral lymphocytes and/or normal autologous mucosa and found no gross rearrangements and deletions. Nevertheless, this possibility cannot be completely ruled out by using one HLA class I probe and few restriction endonucleases. In this context it is also probable that in most HLA class I negative tumours, the regulation of class I HLA antigen expression takes place through normal cellular pathways.

With the exception of melanomas, no significant differences in percentages of

negative cases were noted in the present study between primary and metastatic lesions (table 2). After a comparative analysis of primary tumours and autologous metastases, eight cases of colon cancer metastases showed a decrease in the number of positive cells. We also found metastases originated from positive primary tumours that were negative, and vice versa (table 3). Such observations do not appear to reflect a common phenomenon. Furthermore, tumours showing a relatively frequent loss of antigen expression (17/26 cases of skin basal cell carcinoma) do not produce metastases. Hence, it is possible that in certain tumours the mechanism of immunoresistance to CTLs is mediated by causes other than the decline in class I antigen expression, as has been shown in other tumour systems [2].

HLA class II expression may have a different significance, depending on the tumours. In colon cancer we have noted a correlation between HLA-DR expression and tumour invasiveness: tumours with a more favourable prognosis according to Jass's criteria [22], stages I and II, were positive whereas tumours with the worst prognosis were classified as stages IV and V [20]. Only very well differentiated larynx tumours showed DR expression. However, class II antigen expression in melanoma has been associated with tumoral progression or malignant transformation of melanocytes [1, 25]. An increased expression of class II antigens was found in metastatic melanomas which was related to the location of metastases, e.g. 7/15 ganglionar metastases were positive in comparison with only 1/8 cutaneous metastases. Class II expression on tumours may be induced by a local external stimulus (IFN-γ), or it may be constitutive. In the former case expression would tend to be focal and associated with the presence of lymphocytic infiltrate, whereas in the latter the expression would tend to be homogenous. We believe both aspects to be of importance in the evaluation of class II expression on tumours. In fact, two different mechanisms of HLA class II expression in primary melanomas and metastatic lesions have been proposed [31].

The induction assays performed *in vitro* with IFN-γ show that tumour lines can express class II antigen but these effects may be transient, as is observed with the melanoma line. The dynamics of class II induction by IFN are different for each line analysed. It has been reported that IFN-γ is a better class II inducer than IFN-α [4]. The increase of class II antigen mediated by IFN-γ may improve the T helper lymphocyte recognition of tumoral cells. Although the establishment of a cell line selects all populations from the tumour cell sample, we have always obtained a concordance between the HLA phenotype of the fresh tumour sample and the cell lines established. For instance, line CO3 (figure 2) was derived from a class I positive tumour and we obtained a positive HLA class I expression when this line was typed before the treatment with IFN-γ. In the same context, the M4 HLA class I negative melanoma line was derived from an HLA class I negative tumour sample.

In our opinion, there are a number of factors which should be taken into account when assessing alterations in the expression of histocompatibility antigens on human tumours. In this sense the absence of a strong correlation between the variations studied up to now and the behaviour of the tumour may be more apparent than real. The use of monomorphic monoclonal antibodies against HLA-ABC would seem to be of doubtful value in establishing the actual expression of such antigens because imbalance in class I antigen cannot be detected [25]. As regards future research, we consider that a better understanding of the physiological and pathological factors which regulate MHC antigen expression together with more accurate assessment of expression will bring us closer to identifying the mechanisms responsible for tumour behaviour.

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