Association of Colorectal Tumor Epithelium Expressing HLA-D/DR with CD8-positive T-Cells and Mononuclear Phagocytes

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ABSTRACT

Forty-eight human colorectal adenocarcinomas, removed at different stages of development, have been examined immunohistochemically for the expression of class II molecules and for the relationship of such class II expression to infiltrating leukocytes. Forty-four percent of tumor epithelium samples express class II molecules. This expression is confined to the proteins coded for by the HLA-D/DR subregion although the surrounding infiltrating cells express HLA-D/DR, -DQ, and -DP coded proteins. In addition, there are significantly greater numbers of mononuclear phagocytes and T-cells of the CD8 antibody-positive subset associated with the tumors expressing class II molecules on tumor epithelium compared to the class II-negative tumors. The T-cells appear not to be activated judging by the lack of expression of the receptor for interleukin-2 but the mononuclear phagocytes express CR1, the receptor for the complement component C3b, which suggests that they are stimulated.

INTRODUCTION

The interactions between cells participating in an immune response are restricted by class II or HLA-D region proteins which are expressed by Mphs, B-cells and activated T-cells. The HLA-D region is divided into three subregions named HLA-D/DR, -DQ, and -DP. These molecules have been described on cells other than leukocytes. In particular, epithelium and keratinocytes express class II molecules in tissue immune responses such as autoimmune disorders, graft versus host disease, and bowel disease as well as in other kinds of immunological stimulation, for example, parasite infections and contact sensitivity. This has raised the question as to whether the "inappropriate" expression of class II molecules is an effect of infiltrating leukocytes or independent of these cells. Secondly, there is now interest in the distinctive roles in immune responses that the products of HLA-D/DR, -DQ, and -DP subregions might perform. In this study we have investigated the relationship between expression of class II molecules and immune cells in human colorectal tumors with the objective of defining aspects of immune responsiveness which might affect tumor progression.

MATERIALS AND METHODS

Tissue Samples. Pieces of fresh human colorectal carcinoma were obtained from 48 patients. Blocks were cut vertically with reference to the lumen, through the tumor tissue approximately one third distance from the tumor center. Each block contained tumor, leading edge or margin, and adjacent normal tissue and these areas were reflected in the cryostat sections which were subsequently made. We counted infiltrating cells within the stroma surrounding the islands of tumor epithelium and any infiltrating cells penetrating into this epithelium. We concentrated on areas within the tumor which were within 2-3 mm of the margin, avoiding areas of obvious necrosis which, in any case, were rare in this location. In this study we have not considered those infiltrating cells associated with the stroma found between tumor and normal tissue which is the subject of another study.

The tumors originated in the following locations in the large bowel: rectum, 35; ascending colon, 3; sigmoid colon, 10; cecum, 1. One patient presented with two tumors, one in the rectum and one in the sigmoid colon. They were graded according to Dukes' classification by pathologists at St. Mark's Hospital, London. Stage A and B tumors are confined to the gut wall, whereas stage C tumors have spread beyond the bowel wall into secondary sites. Morphologically normal or uninvolved large bowel, located 15-20 cm from the tumor, was also sampled from each patient. Specimens were snap frozen in liquid nitrogen and 7-μm cryostat sections were made.

Monoclonal Antibodies. The mAbs used to stain the various cell types are described in Table 1. Where possible the designation for clusters of differentiation groups is given (24). Undiluted hybridoma cell supernatant was used in all cases unless otherwise indicated. The specificity of mAb 52 was determined to be HLA-D/DR, DQ by two-dimensional gel analysis of immunoprecipitates from 125I-surface-labeled B-cell line, WT46.4

Immunoperoxidase Staining. Immunohistochemical staining was performed as previously (20). In short, tissue sections were acetone fixed for 10 min at 20°C, washed in PBSA and incubated with 25 μL of the particular mAb for 30 min at 20°C. After washing in PBSA, a second layer consisting of horseradish peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Copenhagen, Denmark; 1:30) containing human serum (1:100) was applied for 30 min at 20°C. After washing in PBSA followed by 0.05 M tris-buffered saline, pH 7.6, the peroxidase label was visualized by incubation with 0.06% 3′,3′-diaminobenzidine (Sigma, Poole, Dorset) in 0.12% H2O2 in Tris-HCl, pH 7.6 for 8 min. Sections were counterstained with Harris' haematoyxlin (BDH, Poole, Dorset) and mounted in DPX mountant (BDH).

Analysis of Cellular Infiltrate. In order to ensure an unbiased analysis of the peroxidase stained cells in colorectal tissue, the following precautions were taken. Samples were identified only by hospital number until the accumulated data was assessed. Secondly, the distribution of specifically labeled cells was examined both qualitatively and quantitatively in all tissue sections by two or three independent observers. For each sample, the cellular infiltrate in both tumor stroma and tumor epithelium was assessed by the enumeration of lymphoreticular cells in each tissue compartment for a minimum of five random fields per section for each observer using a Zeiss E11A graticule containing a grid of 100 mm2, with each field viewed at 63-fold magnification through the grid. A minimum of two blocks of tissue (range, 2-4) were cut for each tumor sample. The lobules of tumor epithelium and connective tissue stroma which were counted were selected at random throughout the tissue sections omitting areas of obvious necrosis and not extending beyond the leading edge or margin of the tumor into the stroma adjacent to normal tissue. Cell numbers were calculated per 1.0 mm2 of tissue (either epithelium or stroma). As unpaired Student's t test was used to compare the number of cells in either tumor epithelium or stroma between Dukes' stages B and C.

RESULTS

Class II Molecules: HLA-D/DR, DQ, and DP Expression. Fresh tissue sections of tumor and accompanying uninvolved colorectal tissue were labeled immunohistochemically with

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1 To whom requests for reprints should be addressed, at Macrophage Laboratory, Imperial Cancer Research Fund, Lincoln's Inn Fields, P.O. Box 123, London, WC2A 3PX, England.

2 The abbreviations used are: Mph, mononuclear phagocyte; IL-2, interleukin-2; mAb, monoclonal antibody; PBSA, phosphate buffered saline without Ca2+ and Mg2+, pH 7.4.

3 A. M. Buckle, manuscript in preparation.

4 Dr. Chris Rudd, ICRF, personal communication.

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**Table 1** Monoclonal antibodies used in this study

<table>
<thead>
<tr>
<th>mAb</th>
<th>Specificity</th>
<th>Reference</th>
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<tr>
<td><strong>Anti-class I</strong></td>
<td></td>
<td></td>
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<td>DA2 and 52</td>
<td>DR/DQ</td>
<td>9, Footnote 5</td>
</tr>
<tr>
<td>TAL.1B5</td>
<td>DR α chains</td>
<td>10</td>
</tr>
<tr>
<td>DA6.147</td>
<td>DR α chains</td>
<td>11</td>
</tr>
<tr>
<td>DA6.164</td>
<td>DR β chains</td>
<td>12</td>
</tr>
<tr>
<td>SDR.4.1</td>
<td>DQw1</td>
<td>13</td>
</tr>
<tr>
<td>2HB6</td>
<td>DQw3</td>
<td>14</td>
</tr>
<tr>
<td>Leu10</td>
<td>DQw2</td>
<td>15</td>
</tr>
<tr>
<td>B7/21</td>
<td>DP</td>
<td>16</td>
</tr>
<tr>
<td><strong>Anti-infiltrating leukocyte T Cells</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>UCHT1</td>
<td>CD3, pan T-cell</td>
<td>17</td>
</tr>
<tr>
<td>Leu 3a/3b</td>
<td>CD4, T-helper/inducer subset</td>
<td>18</td>
</tr>
<tr>
<td>Leu 2a,14</td>
<td>CD8, T-suppressor/cytotoxic subset</td>
<td>18*</td>
</tr>
<tr>
<td>Tac</td>
<td>IL-2 receptor</td>
<td>19</td>
</tr>
<tr>
<td><strong>Mononuclear phagocytes</strong></td>
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<td></td>
</tr>
<tr>
<td>3.9</td>
<td>M, 150,000/95,000, anti-p150,95 on Mph</td>
<td>20</td>
</tr>
<tr>
<td>E11</td>
<td>CD3 receptor, CR1</td>
<td>21</td>
</tr>
<tr>
<td><strong>Other</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LE61</td>
<td>epithelial cytokeratins</td>
<td>22</td>
</tr>
<tr>
<td>W6/32</td>
<td>monomorphic determinants</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>on HLA-A,B,C</td>
<td></td>
</tr>
</tbody>
</table>


**Table 2** Colorectal tumor epithelium expressing class II molecules

<table>
<thead>
<tr>
<th>Dukes' classification</th>
<th>Number of cases</th>
<th>%</th>
<th>Number of cases</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class II-negative</td>
<td></td>
<td></td>
<td>Class II-positive</td>
<td></td>
</tr>
<tr>
<td>Dukes' A + B</td>
<td>18</td>
<td>67</td>
<td>7</td>
<td>33</td>
</tr>
<tr>
<td>Dukes' C</td>
<td>9</td>
<td>33*</td>
<td>14</td>
<td>67*</td>
</tr>
</tbody>
</table>

* Using the statistical test of proportions, these two values are significantly different (P = 0.007).

mAbs specific for class II molecules (Table 1). In Table 2, the status of class II expression on tumor epithelium is listed according to tumor stage. Approximately two thirds of the metastasizing tumor epithelium (Dukes' C) are class II-positive as compared to one third of the epithelium from the nonmetastasizing tumors (Dukes' A and B). Alternatively, it can be stated that there are significantly more Dukes' C tumors containing class II-positive epithelium than there are Dukes' C tumors which have only class II-negative epithelium (P = 0.007). There was no correlation between expression of class II molecules by tumor epithelium and location of the tumor lesion within the large bowel. Thus, of the class II-negative tumors, 19 were located in the rectum, one in the ascending colon, six in the sigmoid colon: of the class II-positive tumors, 15 were located in the rectum, two in the ascending colon, one in the caecum, three in the sigmoid colon, and one jointly in the rectum and sigmoid colon. The epithelial cells from all control or uninvolved colorectal samples were class II-negative. In general, the expression of class II molecules on Dukes' B tumor epithelium when it occurred was patchier and confined to a few lobules, whereas in Dukes' C samples whole tumor lobules were frequently stained in a more dense homogeneous fashion (Fig. 1A).

Because of interest in the roles which different class II subregions might have in immune responses, we next tested the samples with mAbs specific for HLA-D/DR, -DQ, and -DP (Table 1 and Fig. 1). Six samples of either Dukes' A or B and seven samples of Dukes' C tumors were tested with mAbs identifying HLA-DR α and β chains, -DQ, -DP molecules and with mAb LE61 specific for epithelial cytokeratins, in order to positively identify tumor epithelium (Fig. 1C). In all cases the only class II specific mAbs which reacted with tumor epithelium were those labeling HLA-DR α and β chains (Fig. 1A). The infiltrating cells, many of which were Mph, served as an internal control for these reactions as they expressed the products of all three subregions, HLA-DR, -DQ, and -DP (Fig. 1B). Precise quantitation of these cells was not done.

**Class I:** HLA-A,B,C. In the majority of samples, the tumor epithelium reacted with mAb W6/32 in a manner similar to the unininvolved colorectal epithelium surrounding the tumor. In the remaining tumors there was a slight decrease in expression of class I molecules. In only one tumor of the 48 tested was there

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* N. Hogg, unpublished data.

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Fig. 1. Indirect immunoperoxidase labeling of consecutive sections of Dukes' stage C primary colorectal adenocarcinoma (× 750). A, labeling of tumor epithelium and infiltrating cells (arrow, one cell) by mAb DA6.164 specific for the β-chain of the HLA-D/DR molecule. B, labeling of infiltrating cells by mAb B7/21 specific for the HLA-D/DP molecule. C, labeling of same tumor area with a cytokeratin-specific mAb LE61.
Fig. 2. Indirect immunoperoxidase labeling of intratumoral mononuclear phagocytes labeled with monoclonal antibody 3.9 and indicated with a (●). E, small clusters of tumor epithelial cells. × 3500.

Table 3 Infiltrating cells\(^a\) within the epithelium and stroma of HLA-D-positive or HLA-D-negative colorectal tumors

<table>
<thead>
<tr>
<th></th>
<th>T-cells</th>
<th>Mononuclear phagocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CD3 (UCHT1)</td>
<td>CD8 (Leu2a)</td>
</tr>
<tr>
<td>Epithelium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-D-positive</td>
<td>9.1 ± 1.4(^c)</td>
<td>7.5 ± 2.1(^c)</td>
</tr>
<tr>
<td>tumors (N = 21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-D-negative</td>
<td>4.6 ± 1.0(^c)</td>
<td>1.8 ± 0.5(^c)</td>
</tr>
<tr>
<td>tumors (N = 27)</td>
<td>P value</td>
<td>0.021</td>
</tr>
<tr>
<td>Stroma</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-D-positive</td>
<td>54.4 ± 9.4</td>
<td>23.0 ± 8.3</td>
</tr>
<tr>
<td>tumors (N = 21)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>HLA-D-negative</td>
<td>43.5 ± 4.2</td>
<td>13.9 ± 1.6</td>
</tr>
<tr>
<td>tumors (N = 27)</td>
<td>P value</td>
<td>0.39</td>
</tr>
</tbody>
</table>

\(^a\) Figures quoted are the means ± SE of the number of cells/1.0 mm\(^2\) tissue.
\(^b\) Calculations based on 30 rather than 48 samples.
\(^c\) Mean values that are significant using an unpaired Student's t test.

a substantial decrease in class I molecule expression.

T Cells and Monocytes. As the appearance of class II molecules on tissue cells frequently appears to coincide with an ongoing immune response, we investigated whether there were particular features of the infiltrate in the colorectal tumors which correlated with class II expression by the tumor epithelium. Therefore, using specific mAbs (see Table 1) we quantitated total T-cells, the CD4 and CD8 subsets of T-cells, as well as Mph. Fig. 2 shows a high magnification view of Mph surrounding clumps of tumor epithelium, labeled with mAb 3.9 and illustrates the manner in which such cells were enumerated. The numbers of T-cells expressing the IL-2 receptor were also analyzed in addition to the numbers of Mph expressing CR1, the receptor for complement component C3b. These receptors can be considered to be indicators of T-cell and Mph stimulation, respectively (19, 25).

Within the tumor epithelium from the HLA-D/DR-positive samples there were significantly greater numbers of CD8-positive cells compared to the HLA-D/DR-negative samples, which was reflected in total T-cell numbers (CD3-positive cells, Table 3). The CD8-positive cells did not appear to be in a stimulated state because very few cells expressed the IL-2 receptor either in the tumor epithelium or stroma.

There appeared to be little difference in Mph numbers within the epithelium between the two tumor categories. However, in the tumor stroma surrounding the islands of class II-positive compared to class II-negative tumor epithelium, the Mph numbers were significantly increased particularly when labeled with the anti-p150,95 mAb (20). In addition, about 50% of these Mph were expressing CR1 which indicates they they were in a stimulated state (21, 25).

DISCUSSION

In this study, we have observed an association between colorectal tumors expressing class II-positive epithelium, and greater numbers of stromal Mph and intraepithelial T-cells, 80% of which are of the CD8-positive subset. Although the expression of class II molecules on cancerous colorectal epithelial cells has previously been described (26, 27), this paper is the first to observe that in such cases in vivo, it is solely the products of HLA-D/DR subregion which are expressed and not those of the -DP or -DQ subregions.

This raises the question as to whether the expression of HLA-D/DR by tumor epithelium is able to affect any local immune
response. One study has suggested that HLA-D/DR molecules are the main restriction determinants for antigen presentation (28), although antigen specific T-cell lines have been characterized which respond to antigen presented in conjunction with all three HLA-D subregions. The T-cells which are restricted by HLA-D molecules are not CD8-positive cells, associated with class II expression in this study, but the CD4-positive cells which are present in equal number within both HLA-D/DR-positive and -negative tumor epithelium and stroma. CD8-positive cells are usually restricted by HLA-A,B,C or class I molecules and in this study we observed only a slight reduction in tumor epithelium compared to uninvolved colorectal epithelium in levels of class I molecules suggesting that targets for the CD8 cells would still be available. The increased numbers of stromal Mph, which our previous work has shown to have the phenotype of monocytes from the circulation (29), expressed the products of all three HLA-D subregions although precise quantitation was not done in order to assess whether all subregions were equally represented.

Immune responses are thought to cause the induction of HLA-D expression (30). γ-Interferon which is produced by antigen-activated CD4-positive T-cells (31, 32), can be shown, in vitro, to induce some colorectal cell lines to express HLA-D molecules (33, 34). However it is the Mph and CD8-positive T-cells which differed significantly between the two tumor groups and neither cell type are known sources of γ-interferon during an immune response, although apparently CD8-positive cells can be when mitogenically stimulated (35). However, the CD8-positive cells do not appear to be in a stimulated state as judged by lack of expression of the IL-2 receptor. Thus, at present, the expression of class II molecules by tumor epithelium is not explained by the presence of the cellular infiltrate associated with it.

Within the tumors containing class II-positive epithelium the CD8-positive cells comprised 80% of the epithelial T-cell infiltrate. Both the absolute number and percentage are comparable to the intraepithelial CD8-positive cells found in uninvolved colorectal epithelium (29, 36, 37). Epithelium from class II-negative tumors exhibited much lower numbers of CD8-positive cells. This raises the possibility that it is the class II-negative tumor epithelium which is abnormal in having so few CD8-positive cells and that the expression of class II by tumor epithelium permits retention of migrating CD8-positive cells which would otherwise fail to localize in class II-negative tumor epithelium. A similar situation has been observed in the adult rat in which increasing expression of class II by normal intestinal epithelium is correlated with increased numbers of intraepithelial lymphocytes of the CD8-positive type (38). Whether Mph are attracted to the class II-positive tumors as a result of the presence of the CD8-positive cells, factors made by the tumor cells or other conditions within these tumors, is also unknown.

This study has divided human colorectal tumors into two groups with distinct immunological parameters: tumors with HLA-D/DR-positive epithelium with associated CD8-positive cells plus stimulated Mph and tumors with HLA-D/DR-negative epithelium containing significantly fewer CD8-positive cells and Mph. The challenge will be to unravel the biological meaning. Whether these two types of responses can be correlated with the progress of the tumor remains to be determined.

ACKNOWLEDGMENTS

We are grateful to Dr. B. C. Morson and Dr. Jeremy Jass at St. Mark’s Hospital, London, for samples of colorectal tissue and to Professor M. J. R. Healy and Sharon Love (ICRF) for help with the statistical analyses. We thank Drs. Peter Beverley, Julia Bodmer, Walter Bodmer, Mike Crampton, Birgit Lane (ICRF, London), Keith Guy (Western General Hospital, Edinburgh), and Thomas Waldmann (NIH, Bethesda, MD) for their generous gifts of monoclonal antibodies. We acknowledge the excellent technical assistance of Sarah Murdoch (ICRF).

Note Added in Proof

Since this work was submitted an analysis of 9 additional HLA-D/DR positive tumors was carried out using two mAbs specific for HLA D/DP molecules. Within the DR positive epithelium, a few small areas of HLA D/DP positive cells were seen in 3 of these tumors (A. M. Buckle et al., manuscript in preparation). Ghosh et al. (Int. J. Cancer, 38: 459, 1986) also appear to find some expression of HLA D/DP in their colorectal tumors.

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