Influence of Major Histocompatibility Complex Class I and II Antigens on Survival in Colorectal Carcinoma

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ABSTRACT

HLA-A, B, C and HLA-D molecules present antigenic peptides to the antigen-specific receptor of autologous T-lymphocytes. T-cell-mediated host-versus-tumor response might therefore depend on the presence of these molecules on tumor cells, although the absence of HLA-A, B, C determinants on a cell has been shown to increase its susceptibility to lysis by natural killer cells.

To investigate whether the presence or absence of HLA-A, B, C and/or HLA-DR in colorectal carcinomas influences relapse rate and time of tumor-related death, 152 patients who underwent putatively curative surgical treatment were surveyed for a maximum of 65 months (mean, 48 months).

As determined by immunohistochemistry, aberrant reduction or loss of HLA-A, B, C/β2-microglobulin molecules was more frequent in tumors of the proximal colon than of the rectosigmoid (P = 0.032) and in mucinous carcinomas than in nonmucinous ones (P = 0.022). An abnormal induction of the HLA-D-associated invariant chain (Li) was more frequent in Dukes’ A and B than in stage C (P = 0.046). Reduction/loss of HLA-A, B, C/β2-microglobulin was correlated with the absence of HLA-DR (P = 0.024) and Li (P = 0.005). In contrast to the prognostic role of tumor stage and grade, the presence versus the absence of HLA-A, B, C/β2-microglobulin and HLA-DR/Li molecules was not correlated with recurrence rate or survival.

We conclude that in spite of an increasing amount of experimental data suggesting the contrary, the status of HLA-A, B, C and HLA-DR expression in colorectal carcinoma seems to be irrelevant in vivo, regarding survival and growth of residual tumor cells after putatively curative resection of the initial tumor burden.

INTRODUCTION

The MHC3 is a gene sequence on 6p2 encoding several sets of immunoregulatory molecules. Two classes of these are polymorphic and mainly serve as restriction elements of the cellular immune response by interacting with the T-cell antigen receptor of autologous T-cells (1).

MHC class I molecules are polymorphic transmembrane glycoproteins, encoded by a group of closely linked loci, the best known designated as HLA-A, -B, and -C. They are assembled with (monomorphic) β2m. β2m, encoded on chromosome 15, is probably independently regulated but has been found to be necessary for the cell surface expression of class I molecules (2). In cellular immunity, class I molecules are structures which present preprocessed (endogenous) antigen (3, 4) and at the same time act as natural ligands of the CD8 molecule on autologous cytotoxic T-cells, thereby mediating cell-cell adhesion and enhancement of antigen-specific receptor binding (5).

MHC class II determinants, likewise, are heterodimeric transmembrane glycoproteins encoded by a group of closely linked genes, those of the best characterized are designated as HLA-DR, -DP, and -DQ. They are composed of an α-chain with restricted polymorphism and a highly polymorphic β-chain. Intracellularly, the class II αβ-dimer is associated with a third, nonpolymorphic Li encoded by a gene on chromosome 5 (6, 7). Li dissociates from this export complex before the class II molecule appears on the cell surface (8). Class II molecules, too, act as peptide-binding structures. The functions best studied consist of internalizing, processing, and presenting of (foreign) antigen (7, 9) to helper T-cells and class II-restricted cytotoxic T-cells (10) and of physically interacting with the CD4 molecule (11).

Since the first description of a loss of class I determinants in colon carcinoma by Csiba et al. (12), it has been repeatedly confirmed (13–18) that about 10 to 15% of colon carcinomas completely lack reactivity with HLA-A,B,C monoclonal framework antibody W6/32 (19) which recognizes a structural epitope on the intact class I/β2m-complex (20), whereas about 20% of the tumors show a markedly reduced binding of W6/32 within the neoplastic population (21). Based on animal models, it has been suggested that class I-deficient tumor cells might either have a survival advantage by escaping from a putative attack by autologous cytotoxic T-cells (e.g. Ref. 22) or, on the contrary, be more prone to lysis by natural killer cells resulting in a survival disadvantage of such a clone (e.g. Ref. 23). Speculations concerning class I antigen status of a tumor and prognosis are controversial.

The induction of MHC class II antigens (and Li) in colon epithelium affected by all kinds of colitis (24, 25) was also observed in (subsets of tumor cells) in about one-half of all colon carcinomas (12–14, 24–29). Whether the presence of HLA-D antigens in colon carcinoma cells should be regarded as “aberrant expression” or whether the absence of these molecules should be regarded as a “(partial) loss of the constitutive or acquired capacity for class II induction” (14) is still an open question. Nevertheless, since their first description it was thought “conceivable that the presence of HLA-DR-positive tumor cells might augment the immunogenicity of any tumor-specific antigens present and thereby improve prognosis” (27).

To determine the influence of the mode of MHC class I and II expression within the neoplastic population of colorectal carcinoma on disease-free survival and on the risk of tumor-related death, we conducted a prospective study surveying 152 patients who underwent putatively curative surgical treatment.

MATERIALS AND METHODS

Patients. A series of 152 patients whose surgical treatment for colorectal carcinoma was regarded as potentially curative on clinical, para-
clinical, and pathohistological grounds [R resection according to the International Union Against Cancer tumor-nodes-metastasis (TNM) classification (29)] entered the study between January 1, 1984, and September 1, 1985 (Table 1). All patients received standardized follow-up examinations including laboratory tests (routine blood count, serum carcinoembryonic antigen and serum Ca-19-9 levels), X-ray of the chest, computerized tomographic scan of the pelvis and liver, ultrasound examination of the liver and endoscopy of the colon in 3-month intervals during the first 2 postoperative years, and thereafter in 6 month periods. When possible, histological or cytological evidence was obtained to confirm metastatic or recurrent disease. However, characteristic changes in ultra-sound, computerized tomographic scan, or X-ray, preferentially in combination with an elevated carcinoembryonic antigen level, were also accepted. No routine adjuvant chemotherapy or any immunotherapy whatsoever was administered. After diagnosis of tumor recurrence patients received standard systemic fluorouracil or a combination of fluorouracil and leucovorin. Follow-up care was organized by the Surgical Department (P. S.), and all data were entered in the tumor registry. Feedback of results from the examinations to the tumor registry was obtained from all 152 patients by means of a computer-assisted appointment and reminder system. By this system all patients were followed until death or until the end of the observation period (June 1, 1989) of this study. The mean observation time was 48 ± 10.2 (SD) months. The lethal disease complex and its dependency on the underlying neoplastic disease were analyzed taking into account the patient's complete record.

Tumors. Immediately after removal, the entire intestinal specimen was examined by one of us (P. M.) and representative samples of tumor tissue were quick frozen in liquid nitrogen for immunohistochemical investigation. The tumors with primary site and metastatic spread well documented at the time of operation were typed, graded, and staged according to Dukes' and International Union Against Cancer classifications (30–32). The data are listed in Table 1.

Reagents. As mAb to a nonpolymorphic determinant of the HLA-A,B,C/β1m complex, W6/32 (20, 21) was used. To detect HLA-DR molecules, mAb ISCR3 (33) was applied, and mAb VIC-Y1 (34) served as reagent for the detection of li. The Mabs were kind gifts of the originating laboratories. Binding of mAb was detected with a polyclonal anti-biotinylated sheep antibody to mouse immunoglobulins and a streptavidin-biotinylated peroxidase complex (Amersham, High Wycombe, United Kingdom). 3-Amino-9-ethylcarbazole and N',N-dimethyl formamide were obtained from Sigma (St. Louis, MO).

Immunostaining Procedure. MAbs were applied in appropriate dilutions to the serial frozen sections of each tissue block. The biotinylated anti-mouse immunoglobulin antibody was diluted 1:50 in phosphate-buffered saline and to the streptavidin/peroxidase complex 1:100. Incubation times were 1 h at room temperature for the primary antibody, and 30 min for the second- and third-step reagents. Using 3-amino-9-ethylcarbazole as chromogen (0.4 mg/ml in 0.1 x acetate buffer, pH 5.0, with 5% /V',/V-dimethyl formamide and 0.01% H2O2 for 10 min), the peroxidase reaction caused an intense red precipitate. The sections were rinsed in tap water, counterstained with Harris' hematoxylin, and mounted with glycerol gelatin.

Controls. Intrinsinc positive controls for immunoreactivity in each section consisted of stained stromal dendritic cells, histiocytes, and lymphocyte subsets, which indicated at the same time the reliability of the reaction and the maximal staining intensity of this individual reaction. Thus, minor day-to-day variations in staining intensity did not affect evaluation. Each frozen section series contained a negative control without the primary reagent; additionally, a control using irrelevant isotype-matched mAb was carried out in a limited number of tissue sections and excluded nonspecific binding of secondary reagents. In both sets of negative controls, staining was observed in granulocytes in which endogenous peroxidase was not blocked (e.g., by H2O2-methanol) for the benefit of optimal antigenicity, and a faint microgranular cytoplasmic color precipitate in some epithelial areas due to endogenous biotin; these reactivities were disregarded during evaluation, as was the staining observed in areas of tumor necrosis.

Immunohistochemical Evaluation. Staining was evaluated by three of us (P. M., K. K., F. M.). As intrinsic controls allowed a gradation of staining intensity, the actual reactivity of the tumor cells themselves was scored as either strong, weak, or absent. Many tumors contained strongly stained, weakly stained, and nonreactive tumor cells in various proportions; these were scored in a semiquantitative manner. For statistical analysis, the data were divided into three categories for both classes of MHC antigens which, however, were defined separately for HLA-A,B,C and HLA-DR/II determinants. For reasons discussed below HLA-A,B,C/β1m expression within a tumor was regarded as normal when the entire neoplastic population was strongly stained and no unreactive subsets were observed; it was regarded as reduced whenever a subset of unstained tumor cells was detectable and/or the antigenic density (corresponding to staining intensity) was reduced as compared with that of stromal cells; class I antigen expression was considered as last when the tumor cell compartment was unreactive throughout. HLA-DR/II expression within a tumor was regarded as absent whenever the total amount of tumor cells was completely devoid of antigen; in all cases characterized by a patchy or mosaic-like staining pattern, irrespective of the extent of positivity, HLA-DR/II was regarded as partly induced; a tumor was scored as completely induced whenever the whole tumor cell population expressed the antigens, irrespective of minor variations in antigenic density of individual cells or subsets of tumor cells.

Statistical Evaluation. The statistical analysis of the study was carried out by a computer-based Statistical Analysis System. The observation period was closed on June 1, 1989, i.e., 65 months after the first and 45 months after the last patient had entered the study. Recurrence-free and overall survival rates were calculated by the Kaplan-Meier method (35); only tumor-related events were accepted for statistical analysis. For differences between Kaplan-Meier curves the P value was calculated.
for putatively curative resection, all patients in Dukes' stage D had to be excluded.

Expression of HLA-A,B,C/β2m, HLA-DR, and li in Colorectal Carcinoma. Of 152 primary colorectal carcinomas, 85 (55.9%) were found to express HLA-A,B,C/β2m in the manner described

by a log rank test. A χ² test was applied for the analysis of contingency tables.

RESULTS

Basic clinical and pathological data for the colon carcinoma patients are given in Table 1. As a consequence of the selection...
Table 2 Influence of prognostic variables on recurrence and tumor-related death, calculated on the basis of a follow-up ranging from 65 to 45 months (mean, 48 months)

<table>
<thead>
<tr>
<th>Dukes' stage</th>
<th>%</th>
<th>No. of observations</th>
<th>No. of tumor recurrences</th>
<th>No. of tumor-related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>A, B</td>
<td>100</td>
<td>65.8</td>
<td>12</td>
<td>6</td>
</tr>
<tr>
<td>C</td>
<td>52</td>
<td>34.2</td>
<td>16( P = 0.004^*)</td>
<td>10( P = 0.016^*)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Grade</th>
<th>%</th>
<th>No. of observations</th>
<th>No. of tumor recurrences</th>
<th>No. of tumor-related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well differentiated</td>
<td>17</td>
<td>11.2</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>112</td>
<td>73.7</td>
<td>20</td>
<td>10</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>23</td>
<td>15.1</td>
<td>6( P = 0.162)</td>
<td>5( P = 0.049^*)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Type</th>
<th>%</th>
<th>No. of observations</th>
<th>No. of tumor recurrences</th>
<th>No. of tumor-related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonmucinous</td>
<td>105</td>
<td>69.1</td>
<td>18</td>
<td>9</td>
</tr>
<tr>
<td>Mucinous</td>
<td>47</td>
<td>30.9</td>
<td>10( P = 0.232)</td>
<td>7( P = 0.143)</td>
</tr>
</tbody>
</table>

<table>
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<tr>
<th>Localization</th>
<th>%</th>
<th>No. of observations</th>
<th>No. of tumor recurrences</th>
<th>No. of tumor-related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cecum to descending colon</td>
<td>45</td>
<td>29.6</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Cecum sigmoidum</td>
<td>21</td>
<td>13.8</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Cecum rectum</td>
<td>86</td>
<td>56.6</td>
<td>20( P = 0.144)</td>
<td>10( P = 0.727)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>HLA-A,B,C/(\beta_2\m)</th>
<th>%</th>
<th>No. of observations</th>
<th>No. of tumor recurrences</th>
<th>No. of tumor-related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>85</td>
<td>55.9</td>
<td>17</td>
<td>10</td>
</tr>
<tr>
<td>Reduced</td>
<td>53</td>
<td>34.9</td>
<td>9</td>
<td>4</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>HLA-DR</th>
<th>%</th>
<th>No. of observations</th>
<th>No. of tumor recurrences</th>
<th>No. of tumor-related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>68</td>
<td>44.7</td>
<td>15</td>
<td>8</td>
</tr>
<tr>
<td>Partially induced</td>
<td>76</td>
<td>50.0</td>
<td>12</td>
<td>7</td>
</tr>
<tr>
<td>Completely induced</td>
<td>8</td>
<td>5.3</td>
<td>1( P = 0.621)</td>
<td>1( P = 0.790)</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Invariant chain ((\i))</th>
<th>%</th>
<th>No. of observations</th>
<th>No. of tumor recurrences</th>
<th>No. of tumor-related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absent</td>
<td>27</td>
<td>17.8</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Partially induced</td>
<td>103</td>
<td>67.8</td>
<td>19</td>
<td>11</td>
</tr>
<tr>
<td>Completely induced</td>
<td>22</td>
<td>14.5</td>
<td>4( P = 0.999)</td>
<td>3( P = 0.688)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reduction/loss of HLA-A,B,C/(\beta_2\m) combined with absence of HLA-DR/(\i)</th>
<th>%</th>
<th>No. of observations</th>
<th>No. of tumor recurrences</th>
<th>No. of tumor-related deaths</th>
</tr>
</thead>
<tbody>
<tr>
<td>No</td>
<td>143</td>
<td>94.1</td>
<td>27</td>
<td>14</td>
</tr>
<tr>
<td>Yes</td>
<td>9</td>
<td>5.9</td>
<td>1( P = 0.672)</td>
<td>2( P = 0.104)</td>
</tr>
</tbody>
</table>

* Significant.

Survival Analysis. The survival analysis calculated on the basis of 152 patients (Table 2) showed significantly diverging curves of disease free survival and of probability to succumb to tumor-related death for the tumor stage (Fig. 3A). Furthermore, the relative risk of tumor-related death was significantly higher in cases of grade III and IV of tumor differentiation (Fig. 3B). No discriminating effect on both disease-free survival and risk of tumor-related death, however, could be detected when attention was focused on the expressional status of HLA-A,B,C/\(\beta_2\m\), HLA-DR, and \(\i\). Actually, the curves (Fig. 3, C–E) of different levels of these parameters were largely superimposable. In order to test whether the combined modality “reduction/loss of HLA-A,B,C/\(\beta_2\m\) determinants in the absence of HLA-DR/\(\i\)” is a risk factor, the data were analyzed in this respect. Although there was a tendency for these relatively few patients to die earlier due to a tumor-related cause, the level of significance was not attained.

DISCUSSION

Under normal conditions, colon epithelium constitutively expresses HLA-A,B,C antigens and lacks HLA-D/\(\i\) determinants (13, 24, 25, 36, 37). In an inflammatory context, however, MHC antigen expression of cellular tissue components may be profoundly altered, in general by induction and/or enhancement of class II/\(\i\) (38). Inflammation-induced changes in HLA-D/\(\i\) expression have also been observed in colon adenomas and in a major subset of colon carcinomas (26), indicating that the regulatory pathways are unaffected by the process causing neoplastic transformation in such tumors. On the other hand, there is ample evidence that malignant transformation may lead to...
aberrant MHC antigen expression of the tumor cells. Aberrations observed consist of an abnormal reduction or loss of either class I or II antigens, or both, on the one hand (39, 40), and a neo-expression of class II antigens in the absence of inflammatory cells, on the other (13, 25, 41).

Our finding that 9.2% of colorectal carcinomas completely lack class I determinants and another 34.9% show reduced HLA-A,B,C/β2m expression is in good accordance with data reported by Ghosh et al. (14) and ourselves in previous publications on different cohorts (13, 17). In more detailed investigations, the complete loss of W6/32 reactivity could be largely attributed to a defect in regulation of β2m expression as determined by a lack of β2m mRNA and antigen in such tumor cells (42). The reduction of W6/32 reactivity, however, was shown to be due to allelic defects in class I α-chain expression (43–45).

We can furthermore statistically confirm the observation of van den Ingh et al. (15) that mucinous tumors often lack HLA-A,B,C molecules. It was, however, not possible to reproduce
our previous finding (13, 17) of a correlation of aberrant class I expression and poor degree of differentiation; the present data mark a tendency in this direction but fail to be significant. The cohort of Momburg et al. (13) was different from that of Stein et al. (17), but both pilot studies were unselected regarding the extent of radical of surgical treatment. At present, we have no explanation for the statistical accumulation of (in) completely HLA-A,B,C-deficient carcinomas in the proximal parts of the colon.

In agreement with Daar et al. (27), Rognum et al. (29), Csiba et al. (12) and previous studies by ourselves (13, 26), we again observed an induction of HLA-DR in more than one-half of colorectal carcinomas. The neo-expression of the HLA-D-associated invariant chain (li) is even more frequent. As determined by immunostaining of serial tissue sections, the microtopographic extension of li induction always including (or at minimum being identical with) the HLA-DR pattern of induction is strongly suggestive of a sequential induction in the order li → HLA-DR, which seems to be highly conserved in both inflammatory and neoplastic states (24, 37, 40, 46). This sequence of induction can also be generated in vitro in the colon carcinoma cell line HT29 exposed to a combination of γ-interferon and tumor necrosis factor α (47).

Correlating the mode of HLA-DR/li expression with presence and local density of lymphohistiocytic stromal infiltrates in breast carcinoma, Koretz et al. (41) could detect severe dysregulation of these molecules in a subset of tumors, which went in both directions: a hyperexpression which was inadequate as compared to the extent of local inflammation, and the contrary, an inadequate hypoexpression, i.e., a refractory state. Interestingly in this respect, we obtained a correlation of reduction of HLA-A,B,C/β₂m expression with a complete absence of HLA-DR/li molecules in colorectal carcinoma. This nonrandom constellation indicates the presence of a pathological down-regulating signal causing an abnormal expressional MHC status. The same connection emerged in a series of 206 breast carcinomas we investigated (46).

It has been shown in several animal models that the immunogenicity of tumors depends on the expressional status of MHC class I antigens of tumor cells (48). In human in vitro systems using cells of solid tumors and autologous lymphocytes, masking of target cell HLA-A,B,C molecules by mAb W6/32 was found to abrogate cytotoxic T-lymphocyte-mediated cytolysis in only a few experiments (39–51), particularly those in which melanoma cells were exposed to autologous T-cell clones in the presence of interleukin 2 (52–53).

Clinical studies on MHC expressional status of malignant tumors and prognosis are still scarce. In a retrospective study of 50 patients with stage I melanoma, those whose primary tumor was HLA-DR positive had a significantly poorer disease-free survival than those with HLA-DR-negative primaries (54). Likewise, as reported by van Duinen et al. (55), lack of HLA-A,B,C and/or neo-expression of HLA-DR might have contributed to the unfavorable clinical course of a cohort of 39 melanoma patients whose metastases were investigated in this regard. Conversely, in B cell lymphoma patients, the anomalous loss of the constitutively expressed HLA-DR locus products within the neoplastic population was correlated with significantly shorter survival (56). However, since we have shown that defects in HLA-D/li expression in B-cell lymphomas are significantly more frequent in tumors of high-grade malignancy (40), it cannot be excluded that this aberrancy is nothing more than an epi-phenomenon.

In a preliminary study, we concluded from our survival data that reduction or loss of HLA-A,B,C antigens in colorectal carcinoma is not a prognostic parameter (17). This study was based upon 159 unselected patients; maximum follow-up was 39 months, and the survival data were calculated irrespective the cause of death. The mode of HLA-A,B,C expression did not influence survival within this time of observation.

The prospective study presented herein confirms the paramount prognostic implication of disease stage (57–64) and the significance of histological grade (58–60, 64) of the tumor on disease-free survival and on the relative risk of tumor-related death of patients who underwent potentially curative surgical treatment for colorectal carcinoma. Although the relative risk of recurrence and tumor-related death in our group of patients are both in the lower range of data reported in this respect, they are, however, in good accordance, e.g., with the results of the Gastrointestinal Tumor Study Group (57), of Wiggers et al. (61), and of Stähle et al. (63), at least as far as stage Dukes A and B is concerned. It is important to stress these aspects in view of the reliability of our statements on MHC antigen expression and prognosis.

Against this background, our central finding is that in colorectal carcinoma, the presence or absence of both HLA-A,B,C/β₂m and HLA-DR/li has no effect on recurrence nor does the expressional status of these molecules alter the risk of tumor-related death after putatively curative resection of the tumor. Since the residual tumor cell burden is very low under these conditions, a potential T-cell mediated host-versus-tumor reaction should have the chance to be effective. We conclude from our data that the nature of MHC molecules do not profoundly alter survival, possible spread, and growth of such residual tumor cells. The most suitable explanation for our result is that the residual tumor cells are not affected by the T-cell system. This might be due to an absence of tumor-associated antigen on MHC molecules on the tumor cell surface or by T-cell anergy probably mediated via selective suppression or (postthymic?) negative selection of potentially effective T-cell clones. Considering the fact that our immune system is capable of effectively eliminating macroscopic masses of nociferous cells (e.g., in infectious mononucleosis), one is reluctant to accept the idea that it might not be involved spontaneously in the elimination of neoplastic clones. Even if so, immunotherapeutic strategies should be put forward similar to those proposed by Rosenberg et al. (65), but with adjuvant implications, to sensitize and stimulate T-cells in order to control minimal residual disease after successful tumor surgery.

ACKNOWLEDGMENTS

We are indebted to Ingeborg Brandt and Margarete Kaiser for technical assistance, to Doris Hall for organizing the patients' follow-up, to Volker Schwarz and Thomas Gernet for biostatistical help, and to Nicole Beek, M.D., and Peter Martens for help in preparing the manuscript.

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