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*J Immunol* 1998; 161:814-820; ;
http://www.jimmunol.org/content/161/2/814

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*The Journal of Immunology* is published twice each month by The American Association of Immunologists, Inc., 1451 Rockville Pike, Suite 650, Rockville, MD 20852
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Print ISSN: 0022-1767 Online ISSN: 1550-6606.
HLA Class II Expression in Uninducible Hepatocarcinoma Cells After Transfection of AIR-1 Gene Product CIITA: Acquisition of Antigen Processing and Presentation Capacity

Silvia Sartoris,* Maria Teresa Valle,† Andrea De Lerma Barbaro,‡ Giovanna Tosi,§ Tiziana Cestari,* Antonella D’Agostino,‡ Anna Maria Megiovanni,† Fabrizio Manca,† and Roberto S. Accolla²§

The AIR-1-encoded CIITA transcriptional activator is crucial for both constitutive and IFN-γ-induced MHC class II gene transcription. We show here that the MHC class II negative phenotype of the human hepatocarcinoma cell lines Alexander and HepG2 remains unmodified after treatment with IFN-γ, although MHC class I expression is up-modulated. This correlates with absence of CIITA mature transcripts. Transfection of an expressible CIITA cDNA in Alexander cells resulted in a very high cell surface expression of all three human class II subsets, HLA-DR, -DP and -DQ, indicating that normally observed induction of CIITA expression by IFN-γ is probably blocked, in the hepatocarcinoma cell lines, at the level of CIITA transcription and not at the level of IFN-γ receptor binding and signal transduction mechanisms. To assess whether MHC class II expression on CIITA-transfected Alexander cells could have functional relevance, we tested their capacity to present antigenic peptides to an HLA-DR-restricted T cell line specific for a peptide of Mycobacterium tuberculosis Ag85 protein. It was found that the transfected cells could not only present the exogenously supplemented peptide but also process Ag85 protein to generate the specific epitope recognized by the HLA-DR-restricted T cell line. Similar results were obtained with CIITA-transfected CFPAC-1 pancreatic adenocarcinoma cells, which differed from Alexander cells in that they were inducible by IFN-γ. These results suggest new strategies to act on CIITA for increasing the potential of a tumor cell to present putative tumor Ags to the immune system. The Journal of Immunology, 1998, 161: 814–820.

Major histocompatibility complex class II Ags are highly polymorphic cell surface glycoproteins that play a fundamental role in the correct functioning of the immune system. In fact they serve as receptors for antigenic peptides, which only under this form can be correctly presented to the clonotypically distributed specific receptor of regulatory T cells and can trigger the cascade of events leading to both cellular and humoral immune effector functions (1). Both qualitative and quantitative alterations in the expression of the MHC class II molecules dramatically affect the onset and maintenance of the immune response (2), and may be the basis of a wide variety of disease states, such as autoimmunity and immunodeficiency (3, 4). MHC class II molecules, designated HLA-DR, -DQ, and -DP in human and I-A and I-E in mouse, show a restricted tissue distribution being expressed in few cell types, such as B cells, macrophages, thymic epithelial cells, and dendritic cells. Furthermore MHC class II expression can be constitutive, as in B cells, or inducible, as in macrophages, endothelial cell, and in some tumors, by a variety of exogenous stimuli, among which the cytokine IFN-γ certainly constitutes an important member (5, 6).

Most regulation of expression of MHC class II molecules is under the control of transcriptional mechanisms that are both cell type and development specific (7–9). In this context, a pivotal role is played by the CIITA transcriptional activator (10) encoded by the AIR-I locus (11). Defects impairing AIR-I locus function result in failure to transcribe all class II gene subsets, as in the B cell mutant RJ2.2.5 (12), as well as in some B cell lines derived from patients affected by bare lymphocyte syndrome (4).

Expression of CIITA is also required for IFN-γ-inducible MHC class II expression, as demonstrated by the analysis of the AIR-γ-inducible normal cells (13) and cell lines (14–16). Moreover, transfection of CIITA cDNA under the control of a ubiquitous promoter in MHC class II-inducible cells results in a constitutive class II expression (14, 16), confirming and extending previous observations on the dominance of the constitutive class II-positive over the inducible phenotype in somatic cell hybrids (17, 18). Interestingly, terminal differentiation of B cells in plasma cells is accompanied by loss of MHC class II gene expression. This event is mediated by dominant suppressor factors activated and/or expressed in plasma cells that suppress the MHC class II gene transcription (19, 20). It has been recently shown that the developmental extinction of class II expression in plasma cells is accompanied by the lack of expression of the AIR-I gene product, as assessed by the absence of CIITA mRNA in human and mouse plasmacytoma cell lines, as
well as in (B cell × plasma cell) class II-negative somatic cell hybrids. Stable transfection of a constitutively expressible CIITA cDNA under the control of a ubiquitous promoter restored expression of human class II genes in both the plasmacytoma cell model (21), and in the (B cell × plasma cell) somatic cell hybrids (22), demonstrating that the AIR-1 locus is indeed the real target of the active suppression that results in the loss of class II gene expression in plasma cells.

Taken together, these observations demonstrate that the AIR-1 gene-encoded CIITA is a major physiologic regulator of the expression of MHC class II genes.

Tumor cells derived from nonlymphoid tissues sometimes express de novo MHC class II genes or are induced to do so by stimulation with IFN-γ. Although the genetic mechanisms underlying these events are still elusive, it has been shown that in certain tumors de novo expression of MHC class II genes can result in enhanced elimination of the cancer cell by the immune system. It is believed that in these cases tumor cells can serve as APCs for tumor-associated Ags and thus facilitate tumor rejection (23, 24).

Hepatocarcinomas, as their normal cell counterpart (25), do not express class II genes, with few exceptions (26, 27). Moreover, although in some circumstances normal human hepatocytes can be induced to express class II genes both in vivo, as in hepatitis B virus (HBV) infection and in active cirrhosis (28, 29), and in vitro, by IFN-γ (28), most hepatocarcinoma tumors are refractory to induction (26), behaving as constitutive MHC class II-negative cells.

The hepatocarcinoma cell lines Alexander and HepG2 are included in this group. We therefore decided to analyze the pattern of expression of CIITA in Alexander and HepG2 and found that it was not expressed in both cell lines. We then explored the genetic and immunologic effects of stable transfection of an expressible CIITA cDNA and particularly the effects on MHC class II gene expression and the possible functional relevance of this expression.

Materials and Methods

Cell lines and cell surface phenotyping

Alexander and HepG2 are human hepatocarcinoma cell lines (26, 28). CF-PAC-1 is a cell line derived from human pancreatic adenocarcinoma (6). Raji is an MHC class II-positive human B cell lymphoma. They were propagated in RPMI 1640 medium supplemented with 10% FCS and glutamine. Parental cells and transfectants (see below) were analyzed by indirect immunofluorescence and cytofluorometry on an Epics Profile apparatus (Coulter, Hialeah, FL), with a series of mAbs specific for the various subsets of human MHC class II Ags. Relevant to this work were the following reagents: D1–12, specific for human DR Ags; B7/21, specific for human DP Ags; BT 3/4, XIII 358.4 and XIV 466.2, specific for human DP Ags; B10/12, specific for the HLA-DP*15 allele, were then added in 100 L medium to the wells, as described (32, 33). After 2 days, the cultures were pulsed with 0.5 Ci [3H]thymidine (5 Ci/mmol specific activity, Amersham, Buckinghamshire, U.K.) and then harvested 12 h later with a Filtermate 196 apparatus. The dried filters were counted in a Matrix 96 counter (Packard Instrument, Downers Grove, IL).

Results

IFN-γ treatment does not rescue HLA class II Ag expression in two human class II-negative hepatocarcinoma cell lines

The human hepatocarcinoma cell lines Alexander and HepG2 were analyzed for MHC class II surface expression by indirect immunofluorescence and FACS analysis, before and after treatment with IFN-γ. Table I shows the mean values of fluorescence obtained for class II HLA-DR, -DP, and -DQ from the established line specific for pep11 and restricted by the HLA-DR*15 allele were then added in 100 L medium to the wells, as described (32, 33). After 2 days, the cultures were pulsed with 0.5 Ci [3H]thymidine (5 Ci/mmol specific activity, Amersham, Buckinghamshire, U.K.) and then harvested 12 h later with a Filtermate 196 apparatus. The dried filters were counted in a Matrix 96 counter (Packard Instrument, Downers Grove, IL). Results are given as cpm (cpm × 10^3).

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Assessment of CIITA-specific transcripts and analysis of CIITA encoding AIR-1 locus in Alexander and CF-PAC-1 cells

We investigated whether lack of MHC class II cell surface Ags in hepatocarcinoma cell lines could correlate with the absence of specific mature transcripts for the various class II genes. Indeed, analysis of cytoplasmic mRNA demonstrated the absence of specific messages for all the class II subsets (data not shown). We then

3 Abbreviations used in this paper: PPD, purified protein derivative; In, invariant.
CIITA-DEPENDENT CLASS II EXPRESSION AND Ag PRESENTATION COMPETENCE

Table I. Cell surface expression of MHC Ags in Alexander and HepG2 human hepatocarcinoma and CFPAC-1 pancreatic adenocarcinoma cell lines

<table>
<thead>
<tr>
<th>Cells</th>
<th>IFN-γ</th>
<th>FITC</th>
<th>HLA-DR</th>
<th>HLA-DP</th>
<th>HLA-DQ</th>
<th>HLA-A, -B, -C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alexander</td>
<td>−</td>
<td>0.31</td>
<td>0.39</td>
<td>0.37</td>
<td>0.37</td>
<td>16.23</td>
</tr>
<tr>
<td>Alexander</td>
<td>+</td>
<td>0.27</td>
<td>0.28</td>
<td>0.38</td>
<td>0.37</td>
<td>31.42</td>
</tr>
<tr>
<td>HepG2</td>
<td>−</td>
<td>0.15</td>
<td>0.16</td>
<td>0.16</td>
<td>0.17</td>
<td>13.59</td>
</tr>
<tr>
<td>HepG2</td>
<td>+</td>
<td>0.16</td>
<td>0.16</td>
<td>0.16</td>
<td>0.15</td>
<td>51.49</td>
</tr>
<tr>
<td>CFPAC-1</td>
<td>−</td>
<td>0.45</td>
<td>0.43</td>
<td>0.38</td>
<td>0.38</td>
<td>10.55</td>
</tr>
<tr>
<td>CFPAC-1</td>
<td>+</td>
<td>0.50</td>
<td>10.90</td>
<td>5.13</td>
<td>7.64</td>
<td>82.41</td>
</tr>
</tbody>
</table>

*Values are expressed as mean fluorescence of the corresponding cells when stained with an irrelevant antibody (FITC) or with Ab specific for the HLA Ag listed on top of the table. Cells were analyzed before (−) or after treatment (+) with IFN-γ.

FIGURE 1. Analysis of CIITA-specific transcripts as detected by RT-PCR using CIITA-specific primers. RT-PCR was conducted by using total cytoplasmic RNA prepared from the various cell lines listed in the top of the figure. Raji, human B cell lymphoma; Alexander, human hepatocarcinoma; CFPAC-1, human pancreatic adenocarcinoma. Cells treated with 1000 U/ml of recombinant human IFN-γ (IFN-γ lane) or transfected with CIITA (CIITA) are indicated. As control, specific transcripts of the housekeeping gene β-actin amplified from the same RNA preparations of the various cells are shown.

analyzed whether lack of class II gene transcription correlated with absence of expression of the AIR-1 gene product CIITA. Figure 1 shows the results of RT-PCR performed on cytoplasmic RNA of Alexander cells, either untreated (Alex lane) or after treatment with IFN-γ (Alex/IFN-γ lane). It can be seen that, in both conditions, no CIITA-specific mRNA was detected in Alexander cells. Similar results were obtained in HepG2 cells (data not shown). As expected, CIITA-specific transcripts were instead expressed in the CFPAC-1 pancreatic adenocarcinoma cells after treatment with IFN-γ (lane CFPAC-1/IFN-γ) but not in untreated cells.

To rule out the possibility that lack of CIITA expression was due to extensive alteration of the AIR-1 gene, analysis of Alexander genomic DNA was conducted. The BamHI restriction enzyme digestion pattern of Alexander genomic DNA did not show any difference when compared either with that of the IFN-γ-inducible CFPAC-1 cells or to that of the constitutive CIITA-expressing Raji cells. Under the above experimental conditions, the probe hybridized with two major fragments of human genomic DNA of about 15 and 9 kb, respectively, and a minor band of 5.6 kb (data not shown).

De novo expression of class II, In chain and DMB genes and up-regulation of DMA gene in Alexander cells transfected with an expressible CIITA cDNA

Introduction of a CIITA cDNA clone under the control of a ubiquitous promoter in Alexander cells generated transfectants expressing CIITA-specific transcripts. The CIITA expression pattern of a representative transfectant is shown in Figure 1 (Alex/CIITA lane) as compared with a similarly raised CIITA transfectant of CFPAC-1 pancreatic adenocarcinoma cells. In this latter case, no difference was observed between IFN-γ-induced and CIITA-transfected cells in the amounts of CIITA-specific transcripts. As shown in Figure 2, both CIITA-transfected cell lines, Alexander and CFPAC-1, expressed de novo at the cell surface all three class II subsets HLA-DR, -DP, and -DQ, at levels comparable with the MHC class II-positive human B cell Raji.

The expression status of In chain, DMA and DMB genes whose products are associated to class II molecules during distinct phases of class II intracellular transport and whose expression may be influenced by CIITA were also analyzed (see Fig. 1). Alexander hepatocarcinoma cells did not express appreciable amounts of either In chain or DMB mRNA, whereas they expressed low amounts of DMA transcripts. This pattern was unchanged after IFN-γ treatment. On the other hand, uninduced CFPAC-1 cells expressed substantial amounts of In chain and DMA transcripts and low but appreciable amounts of DMB mRNA. After IFN-γ treatment, In chain, but not DMA or DMB, specific mRNA was increased. Transfection of CIITA in Alexander cells resulted in a de novo expression of high amounts of In and DMB mRNAs and in substantial increase in the DMA-specific transcripts. Transfection of CIITA in CFPAC-1 cells resulted in In chain mRNA increase comparable with that obtained after IFN-γ treatment, in further increase of DMA-specific mRNA, and in a strong increase of DMB transcripts, especially when compared with the CIITA-constitutive Raji B cell line.

These results demonstrate that the lack of MHC class II gene expression in human hepatocarcinoma cell Alexander, both in unstimulated and IFN-γ-stimulated conditions, is due to the impossibility to transcribe the AIR-1 gene and by consequence to express the corresponding CIITA trans-activator. They also demonstrate that although In chain, DMB, and, to a lesser extent, DMA genes can be up-regulated by CIITA, their expression is not necessarily dependent on the presence of the trans-activator as shown in CFPAC-1 cells.

Ag processing and presentation capacity of CIITA transfectants

To assess whether de novo expression of MHC class II genes on CIITA-transfected cells had functional relevance, we tested their
capacity to present mycobacterial Ags to DR-restricted PPD-specific CD4+ T cell lines. These lines both respond to a specific epitope included in a peptide (pep11) spanning the region between aa 91 to 108 of Ag85. The first T cell line is restricted by the HLA-DR*15 allele (present in Alexander cells); the second cell line is restricted by the HLA-DR*11 allele (present in CFPAC-1 cells).

As shown in Figure 3, the MHC class II-positive CIITA-transfected Alexander cells pulsed with pep11 (ALEX-CIITA, Fig. 3A) stimulated the pep11-specific T cell line, indicating Ag-presenting competence. This ability was dependent on the newly acquired MHC class II expression since the T cell line did not proliferate in response to the untransfected, MHC class II-negative Alexander cells pulsed with pep11 (ALEX, Fig. 3A). In addition, T cells were stimulated by whole PPD (ALEX-CIITA, Fig. 3B) to a similar extent as by pep11, suggesting also an Ag-processing function of the CIITA-transfected cells. However, since PPD is a poorly defined mixture of mycobacterial Ags, it may include degraded antigenic fragments that could be presented by the restriction element independently of processing. To confirm the Ag-processing capacity of CIITA-transfected Alexander cells, we performed additional experiments by using the protein Ag Ag85, a 30- to 32-kDa mycobacterial protein purified over ion-exchange and gel filtration columns to exclude contamination by low m.w. fragments (31). Figure 3 shows that, also in this case (ALEX-CIITA, Fig. 3C), pep11-specific T cells can proliferate to similar extent as when pep11 or PPD were used.

These results show that the CIITA-transfected MHC class II-positive hepatocarcinoma not only can present the offered peptide but also can internalize and process the native protein to generate HLA-DR*15-restricted immunogenic peptides. Similar, although not superimposable, results were obtained when the CIITA-transfected CFPAC-1 pancreatic adenocarcinoma cells were used as APC (Fig. 3, D, E and F). Both the exogenously provided peptide (Fig. 3D) and the whole purified protein (Fig. 3F) were able to stimulate the proliferation of the T cell line although to lesser extent as compared with CIITA-transfected Alexander cells, indicating also in this case acquisition of Ag-processing and presentation competence. It must be stressed that the CFPAC-1 cells used in this experiments displayed a class II-positive phenotype in only 70% of the population.

It must be noted that the Ag-processing and presentation capacity of CIITA-transfected Alexander and CFPAC-1 cells resulted, at plateau, in a reduced efficiency of T cell stimulation when compared with professional APCs.

**Discussion**

The rationale of this work was twofold, first to investigate whether the constitutive MHC class II-negative phenotype of human hepatocarcinoma cells could be modified into a class II-positive phenotype by introduction of an expressible human CIITA-specific cDNA, and second, if this were the case, whether de novo expression of human class II genes in the tumor cells could correlate with
acquisition of immune-related functions such as Ag processing and presentation to T cells.

As far as the first point is concerned, the results presented in this work clearly demonstrate that two distinct human hepatocarcinoma cell lines, Alexander and HepG2, do not express human class II genes because they do not express the classical II \textit{trans}-acting activator CIITA encoded by the \textit{AIR-1} locus. Moreover, stimulation with IFN-\(\gamma\), the most potent inducer of MHC class II gene expression through the activation of CIITA expression, as shown previously (13, 14, 16) and confirmed here on the pancreatic adenocarcinoma cell line CFPAC-1, was unable to rescue class II gene expression in the hepatocarcinoma cell lines. Again, this correlated with inability to express CIITA. This event was not due to defects in IFN-\(\gamma\)-receptor binding and/or cytoplasmic signal transduction pathways, since the treatment with the cytokine could substantially increase the expression of other cell surface molecules such as MHC class I molecules. Thus, it is likely that, in the hepatocarcinoma cells analyzed in this study, the impossibility to express CIITA and, by consequence, MHC class II genes is related to either a developmental block or to a tumor-induced defect affecting specifically the expression of the CIITA-encoding \textit{AIR-1} gene.

Whatever the mechanism, it must affect the transcription of the \textit{AIR-1} gene. Indeed, transfection of Alexander cells with a CIITA-specific cDNA under the control of a ubiquitously expressible promoter, resulted in expression of CIITA mRNA and de novo expression of MHC class II genes and corresponding molecules at high levels. It will be therefore very important to pursue the analysis of the molecular mechanisms regulating the expression of the \textit{AIR-1} gene, the structure of the \textit{AIR-1} gene promoter region (34, 35), its accessibility to specific transcription factors and the mode of action in various cell types, and particularly in tumor cells. Indeed, recent findings have strongly suggested that two promoter sequences seem to control the constitutive \textit{AIR-1} gene expression in B cells and dendritic cells whereas a distinct promoter sequence is involved in the IFN-\(\gamma\)-induced expression (34).

The fact that, upon CIITA transfection, MHC class II genes are strongly expressed further reinforces the notion that neither the chromatin accessibility nor the function of the transcription factors required for a correct expression of class II genes is defective in the hepatocarcinoma cells.

Recently it has been demonstrated that, besides the peculiar function as main switch for the expression of MHC class II genes, in several cellular systems CIITA \textit{trans}-activator may induce or up-regulate other genes such as HLA-DMA, HLA-DMB, and invariant chain (30, 36). Interestingly, all these gene products are strongly involved in Ag processing and presentation to T cells. Thus, classical professional APCs, such as B cells and macrophages, may be strongly dependent on expression of CIITA to exert their function. The hepatocarcinoma cells analyzed in this study did not express CIITA; thus they could not express MHC class II. In addition, the hepatocarcinoma cell line Alexander did not express \textit{In} chain and \textit{DMB} genes and expressed very low amounts of DMA transcripts. This expression pattern was not modified by treatment with IFN-\(\gamma\), but it was drastically changed by CIITA transfection. Indeed, both \textit{In} chain and \textit{DMB} genes were...
strongly induced, and DMA expression was up-regulated. However, expression of CIITA was not necessary to induce the expression of either In chain or DM genes. In fact, as demonstrated by the present study, a CIITA-negative but IFN-γ-inducible tumor cell line of pancreatic origin, CFPAC-1, constitutively expressed substantial amounts not only of In chain but also of DMA and DMB transcripts, although transfection of CIITA could further increase the expression of all the three genes.

Taken together, these results give further support to the notion that CIITA is involved in the coregulation of a variety of genes whose products are important for a correct Ag presentation function. Nevertheless they also establish that the role played by CIITA in the regulatory mechanism of In chain and DM gene expression is certainly distinct and less fundamental than the one played in the regulation of MHC class II gene expression. Therefore, if MHC class II gene expression in tumor cells is required to present putative tumor-associated Ags to T cells and trigger antitumor immune responses (23, 24), suppression of CIITA could be an important mechanism for neoplastic cells to escape immune control.

Based on the above considerations, we therefore asked whether CIITA-transfected MHC class II-positive tumor cells could acquire immunologic competence. The results presented in this study clearly demonstrate that CIITA-mediated de novo expression of MHC class II genes and corresponding proteins in both an IFN-γ-uninducible and an IFN-γ-inducible tumor cell line is sufficient to impart Ag presentation capacity to DR-restricted, PPD peptide-specific T cell lines, not only when tumor cells are pulsed with the relevant peptide but also when they are pulsed with native protein. Thus, CIITA-transfected Alexander hepatocarcinoma and CFPAC-1 pancreatic adenocarcinoma cells can perform both Ag processing and presentation functions in absence of IFN-γ induction. This result is at variance with a recently published observation indicating that CIITA-mediated expression of class II genes in a class II-negative, but IFN-γ-inducible, melanoma cell line is sufficient to impart peptide Ag presentation but not native protein Ag-processing capacity (37). Interestingly, in the above report, treatment with IFN-γ of both CIITA-transfected and untransfected melanoma cells resulted in Ag presentation capacity when either the peptide or the native protein Ag were used, suggesting that in this tumor model activation of Ag-specific T cells requires additional CIITA-independent factors inducible by IFN-γ. The reasons for the discrepancy between these results and our results are not known, but they may be due to the different cellular systems utilized in the two approaches. In fact, that CIITA alone can be sufficient to impart Ag presentation capacity was also recently reported by Armstrong and collaborators (38), who have transfected MHC class II-negative murine sarcoma cells with the trans-activator and shown that tumor cells express class II molecules and corresponding proteins in both an IFN-γ-uninducible and an IFN-γ-noninducible MHC class II-negative phenotype, such as the one displayed by most hepatocarcinoma cell lines, is due to lack of expression of the CIITA gene product to be down-modulated in those disease states characterized by excessive and unwanted Ag presentation to the immune system (38), or, alternatively, as target gene product to be down-modulated in those disease states characterized by excessive and unwanted Ag presentation to the immune system, as in some autoimmune diseases.

Acknowledgments

We thank Dr. Antonio Amoroso and Dr. Sergio Curtori, Center of Immunohistocompatibility of Turin, for HLA typing of Alexander and CFPAC-1 cells. R.S.A. thanks Dr. Lorenzo Moretta, IST, University of Genova, for advice and continuous support to his research.

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