MHC Class II Engagement in Brain Endothelial Cells Induces Protein Kinase A-Dependent IL-6 Secretion and Phosphorylation of cAMP Response Element-Binding Protein

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Activated endothelial cells can directly participate in immune responses by interacting with immunocompetent cells via class II MHC proteins. We show here that, after induction of MHC class II molecule expression by IFN-γ, rat brain endothelial cells responded to MHC class II ligands, anti-MHC class II Abs, or superantigens by expression of IL-6 transcript and IL-6 secretion. This response was not affected by protein kinase C depletion but was mimicked by the cAMP-elevating agent forskolin and completely blocked by H89, an inhibitor of cAMP-dependent protein kinase (PKA). Involvement of a cAMP/PKA signaling pathway in response to MHC class II ligands was further demonstrated by measurement of a dose-dependent increase in cAMP level and phosphorylation of the transcription factor cAMP response element-binding protein (CREB). Our results indicate that MHC class II engagement in brain endothelial cells is directly coupled to IL-6 production via a cAMP/PKA-dependent intracellular pathway. The Journal of Immunology, 1999, 163: 3636–3641.

Activated endothelial cells can directly participate in immune responses by interacting with immunocompetent cells via class II MHC proteins. We show here that, after induction of MHC class II molecule expression by IFN-γ, rat brain endothelial cells responded to MHC class II ligands, anti-MHC class II Abs, or superantigens by expression of IL-6 transcript and IL-6 secretion. This response was not affected by protein kinase C depletion but was mimicked by the cAMP-elevating agent forskolin and completely blocked by H89, an inhibitor of cAMP-dependent protein kinase (PKA). Involvement of a cAMP/PKA signaling pathway in response to MHC class II ligands was further demonstrated by measurement of a dose-dependent increase in cAMP level and phosphorylation of the transcription factor cAMP response element-binding protein (CREB). Our results indicate that MHC class II engagement in brain endothelial cells is directly coupled to IL-6 production via a cAMP/PKA-dependent intracellular pathway. The Journal of Immunology, 1999, 163: 3636–3641.

Antigen-specific activation of CD4+ T lymphocytes is mediated by interaction between the TCR and MHC class II-peptide Ag complex. Although constitutive expression of MHC class II genes is restricted to "professional" APCs like B cells and monocytes, expression can also be induced, in inflammatory situations, in a variety of MHC class II-negative cells, such as smooth muscle cells, fibroblasts, keratinocytes, and endothelial cells (1, 2). Accordingly, MHC class II expression has been reported in brain microvessel endothelial cells during inflammatory diseases of the CNS (3), like multiple sclerosis and its animal model experimental encephalomyelitis (3, 4) or, in vitro, after IFN-γ treatment (5). Induced expression of MHC class II molecules on these cells allows them to contribute to the recruitment and activation of cytotoxic CD4+ T lymphocytes in the brain (6).

The capacity of MHC class II molecules to transduce signals was initially suggested by studies demonstrating that anti-MHC class II mAbs can induce cellular response in human hemopoietic cells. Alternatively, TCR activation can proceed after MHC class II engagement by microbial superantigens, which bring TCR and MHC class II in close proximity by binding to amino acid residues outside of the conventional Ag-binding groove, on both Vβ and MHC class II molecules (7). Indeed, binding of microbial superantigens, such as staphylococcal enterotoxins (SE) α, β, or ε toxic shock syndrome toxin 1 (TSST-1), to MHC class II molecules was initially demonstrated by their ability to stimulate many T cells, in both mice and humans, in the presence of MHC class II-positive cells (for review, see Ref. 7). Although binding of SE to MHC class II molecules appears to be much less restricted than to Vβ, important differences in their binding affinity and specificity to various isotypes and allotypes have been reported in mice and humans (8, 9).

In response to MHC class II ligands, downstream signaling occurs through MHC class II molecules, resulting in cytokine secretion and/or gene transcription. Such events have been reported in B lymphocytes (10, 11) or in cells induced to transiently express MHC class II molecules, such as synoviocytes and keratinocytes (12, 13). The signaling pathways involved depend on cell type or activation level. Surprisingly, little is known about endothelial cell response to MHC class II engagement.

In the present study, we used the nontransformed immortalized rat brain microvessel endothelial RBE4 cells, which have been extensively characterized by us and others and maintained in culture the phenotype of the blood-brain barrier endothelium (14–19). We report that in RBE4 cells, MHC class II engagement by mAb or microbial superantigens induces an increase in cAMP level that leads to transcriptional regulation and secretion of IL-6. This is the first demonstration that MHC class II molecules ectopically expressed on brain endothelial cells can transduce intracellular signals leading to cytokine production and suggest that their engagement with TCR directly contributes to gene regulation in endothelial cells.

Materials and Methods

Reagents

Recombinant rat IFN-γ was purchased from Life Technologies (Ergny, France). Human IL-1β and TNF-α and mouse IL-6 were from Genzyme.
RBE4 cells were grown on type I collagen-coated dishes in medium/ Ham's F-10 (1:1; Life Technologies Gibco-BRL, Ergany, France), supplemented with 10% FBS and 1 mg/ml basic fibroblast growth factor (Boehringer Mannheim, Mannheim, Germany), and used between the 30th and 60th passage. RBE4 cells were seeded at 10^5 cells/cm^2 in 6-well dishes and incubated for 3 days at 37°C, 5% CO_2 humidified incubator, until confluent. Cytokine or LPS treatments were performed in fresh medium for 24 h; cell supernatants were then collected and processed for IL-6 assay as described above. Alternatively, cells were pretreated for 48 h in presence of 100 U/ml IFN-γ to induce the expression of MHC class II molecules, before incubation in presence of OX6 Ab or bacterial superantigens followed by IL-6 bioassay, PCR analysis, or cAMP accumulation assay.

Flow cytometry analysis
Subconfluent RBE4 cells were incubated in the presence of increasing concentrations of recombinant rat IFN-γ, for 4 h in fresh complete medium. Single-cell suspensions were prepared by short trypsin-EDTA treatment and cells were incubated in 100 μl PBS containing 3% FBS and 0.1% sodium azide, at a final density of 2 × 10^5 cells/ml, in the presence or absence (negative control) of OX6 Ab for 30 min on ice. Afterward, cells were washed in the same buffer and resuspended in 100 μl in the presence of FITC-labeled goat anti-mouse IgG for another 30 min on ice. For the rat IL-6 cDNA amplification, a sense primer (5′-CAAGAGACTTCCAGCCAGTTGC-3′) corresponding to nucleotides 81–102 and an antisense primer (5′-TTGCCGAGTAGACCTCATAGTGACC-3′) corresponding to nucleotides 694–670 were purchased from Clontech (Palo Alto, CA). As an internal standard of reverse transcriptase efficiency, a set of primers specific for the human G3PDH were purchased from Clontech.

All pairs of primers hybridize to different exons, allowing detection of contaminating genomic DNA in the cDNA preparations. Amplification products were electrophoresed through a 2% agarose gel and blotted onto nylon membrane. The membrane was prehybridized at 42°C for 24 h at 42°C in 4× SSC, 5× Denhardt’s solution, 40% deionized formamide, 50 mM sodium phosphate (pH 6.8), and 100 μg/ml heat-denatured salmon sperm DNA. Hybridization was performed in the same solution in the presence of 32P-labeled murine IL-6 CDNA probe, a 1.1-kb EcoRI restriction fragment of pHPIB, kindly provided by Dr J. Van Snick (Ludwig Institute for Cancer Research, Brussels, Belgium). Final washes were at 42°C in 1× SSC, 0.1% SDS.

cAMP accumulation assay
RBE4 cells were grown until they reached confluence in 6-well dishes and pretreated for 48 h in the presence of 100 U/ml IFN-γ. Cells were lysed in cold ethanol for 10 min. Amounts of cAMP were quantified with an Amershams (Les Ulis, France) cAMP ELISA determination kit.

Immunoblot analysis
After treatment, cells (10^5) were washed in ice-cold PBS and then immediately lysed in 80 μl SDS-sample buffer. Cellular extracts were then loaded on polyacrylamide gel and submitted to electrophoresis, as previously described (19). For serial incubation of membranes, bound Abs were stripped out by incubation for 10 min in 0.1 M glycine, pH 2.5, and the membranes were reprobed with different Abs as described above.

Results
Surface expression of MHC class II molecules
To identify the conditions for MHC class II expression on RBE4 cell surface flow cytometry analysis was assessed with the use of OX6 mAb. As shown in Fig. 1, RBE4 cells did not constitutively express MHC class II molecules. During inflammatory diseases of the CNS, IFN-γ concentration largely increases until reaching >1000 U/ml in cerebrospinal fluid (22, 23). Therefore we determined MHC class II expression in IFN-γ-treated RBE4 cells. MHC class II expression was detected on virtually all RBE4 cells after 48 h treatment with 50 U/ml IFN-γ (Fig. 1A). Higher concentrations of IFN-γ further increased MHC class II surface expression (Fig. 1B).

Induction of IL-6 secretion by MHC class II ligands
On the basis of these data, RBE4 cells were pretreated for 48 h with IFN-γ (100 U/ml), before incubation for 24 h in the presence of OX6 mAb. Treatment with OX6 mAb (10 μg/ml) induced IL-6 secretion that could be detected as early as 4 h after stimulation. The maximum level of IL-6 secretion induced by OX6 mAb treatment for 24 h was higher than that those obtained in response to IL-1β, TNF-α, IFN-γ, or LPS (Table 1) and was not further increased by OX6 cross-linking with a second Abs (data not shown).

SEs have been well characterized as human, murine, and rat MHC class II ligands, and their potential ability to induce IL-6 secretion by RBE4 cells was investigated. Treatment with 10 μg/ml SEA induced IL-6 secretion by IFN-γ-activated RBE4 cells at a slightly higher level than treatment with OX6 mAb (Fig. 2A). SEE and SEB also induced IL-6 secretion, although to a lower extent, whereas TSST-1 had no effect. As expected, no significant secretion was detected when RBE4 cells were not pretreated with IFN-γ (<10 pg IL-6/10^6 cells), and thus did not express MHC class II molecules (Fig. 2A). No secretion of IL-6 was detected when IFN-γ-pretreated cells were left untreated during the 24-h incubation time of the assay (Fig. 2A, NT) or were treated with an Ab directed toward an unrelated adhesion molecule, ICAM-1 (not shown). As reported in Fig. 2B, these effects were dose dependent, between 0.5 and 10 μg/ml of MHC class II ligands. IL-6 secretion was detectable 4 h after MHC class II engagement and increased until 24 h of incubation (Fig. 2C).

Taken together, these results indicate that engagement of MCH class II molecules on brain endothelial cells can induce IL-6 secretion.
Induction of IL-6 mRNA by MHC class II ligands

To assess whether regulation of IL-6 secretion occurs at the transcriptional level, RT-PCR analysis was performed on total RNA samples from unstimulated, OX6-stimulated, or SEA-stimulated RBE4 cells at different time points. As shown in Fig. 3, a cDNA fragment of the expected size (614 bp) and hybridizing with an IL-6 specific probe was amplified as soon as 2 h after treatment with either OX6 or SEA and was still detected at 24 h. G3PDH amplification was used as an internal standard. These data establish that the induction of IL-6 production by MHC class II ligands in RBE4 cells is correlated with the elevation of IL-6 mRNA level in RBE4 cells.

Role of protein kinase C (PKC) and PKA in MHC class II-induced IL-6 secretion

Previous studies addressing the molecular basis of MHC class II signaling in B cells have pointed out the role of PKC and intracellular cAMP (24–26).

In RBE4 cells, the PKC activator PMA (160 nM) appeared as a weak inducer of IL-6 secretion (data not shown). Furthermore, cell pretreatment for 16 h with 160 nM PMA, a condition known to deplete PKC in various cell type (27) including RBE4 cells (data not shown), failed to affect the capacity of OX6 mAb to induce IL-6 secretion. These observations strongly suggest that the OX6-associated signaling pathway leading to IL-6 secretion is PKC independent.

A potential role of PKA was investigated by comparing RBE4 cell responses to MHC class II ligands and to forskolin, a direct activator of adenyl cyclase and PKA pathway. As shown in Fig.

Table 1. IL-6 induction in RBE4 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>IL-6 (pg/10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NT</td>
<td>&lt;10</td>
</tr>
<tr>
<td>IL-1β (500 U/ml)</td>
<td>182 ± 13</td>
</tr>
<tr>
<td>TNF-α (100 ng/ml)</td>
<td>267 ± 24</td>
</tr>
<tr>
<td>IFN-γ (50 U/ml)</td>
<td>239 ± 10</td>
</tr>
<tr>
<td>LPS (10 µg/ml)</td>
<td>335 ± 35</td>
</tr>
<tr>
<td>OX6 mAb (10 µg/ml)</td>
<td>1669 ± 241</td>
</tr>
<tr>
<td>IgG1 mAb (10 µg/ml)</td>
<td>&lt;10</td>
</tr>
</tbody>
</table>

Treatment of RBE4 cells was performed during 24 h. Results are representative of three independent experiments, performed in six replicates.
A, forskolin induced IL-6 secretion in a dose-dependent manner. In addition, the highly selective PKA inhibitor H89 (28) completely blocked not only the forskolin-induced IL-6 secretion but also IL-6 secretion induced by MHC class II ligands, OX6 or SEA (Fig. 4B). At the concentrations used (30–60 μM), H89 did not affect the viability of RBE4 or 7TD1 cell lines and did not induce per se any IL-6 secretion by RBE4 cells (Fig. 4B).

These results indicate that PKA activation is sufficient to induce IL-6 secretion and is necessary for MHC class II-induced IL-6 secretion by RBE4 cells.

To further demonstrate the induction of the PKA pathway by MHC class II ligands, cAMP level quantification was performed in RBE4 cells. OX6 as well as SEA induced cAMP accumulation in a dose-dependent manner (Fig. 5): cAMP level was maximum after treatment with 10 μg/ml OX6 mAb or SEA. Interestingly, a similar level was measured in response to 10^{-7} M forskolin, all three effectors also inducing, at those concentrations, similar levels of secreted IL-6 (Fig. 5). No cAMP accumulation was detected when IFN-γ pretreatment was omitted or when cells were treated with irrelevant isotype-matched Abs (not shown).

These results demonstrate that MHC class II engagement induces cAMP accumulation in RBE4 cells and strongly suggest that this is the major pathway responsible for MHC class II-induced IL-6 secretion.

MHC class II stimulation induces phosphorylation of the cAMP response element-binding protein (CREB)

Because the IL-6 promoter is under the control of a cAMP response element (29), we intended to assess the role of CREB in the response to MHC class II engagement. The ability of CREB to activate transcription in response to cAMP is regulated by its phosphorylation at residue Ser133 (30). Western blot analysis of RBE4 cell extracts from untreated or OX6 (or SEA)-treated cells was thus performed using Abs that specifically recognize the Ser133-phosphorylated form of CREB. CREB was found to be phosphorylated within minutes of MHC class II stimulation by either OX6 or SEA (Fig. 6A, upper panels). Phosphorylation reached a maximum between 5 and 10 min after binding and then gradually decreased over the next 20 min. The same blot was subjected to Western blot analysis by using anti-CREB Abs that do not discriminate between phosphorylated and unphosphorylated forms (Fig. 6A, lower panels), indicating that similar CREB quantity was loaded in all lanes. In contrast, when cells were treated with an irrelevant isotype-matched Ab, no change in CREB phosphorylation was observed (not shown).

OX6 cross-linking did not induce significantly stronger CREB phosphorylation than treatment with OX6 alone (data not shown). Pretreatment of the cells with the specific PKA inhibitor H89 prevented the phosphorylation of CREB by MHC class II engagement, as well as by forskolin (Fig. 6B, upper panels), illustrating the major role of PKA in MHC class II-induced CREB phosphorylation. In the same conditions, increase in cAMP level was still observed, confirming the viability of the cells and their ability to respond normally to stimulation (not shown).
These results indicate that the PKA-dependent IL-6 secretion observed in response to MHC-class II engagement is associated with CREB phosphorylation.

Discussion

In our study, we demonstrate that after MHC class II induction by IFN-γ, MHC class II ligands induce IL-6 secretion by brain endothelial RBE4 cells. Moreover, we show that this response occurs via a cAMP/PKA-dependent pathway and is associated with phosphorylation of the transcription factor CREB.

These data indicate differences between biological activity of the various superantigens tested, which might be directly related to variations in fine specificity of binding. Our results suggest that cross-linking of MHC class II molecules is not required for induction of MHC class II-coupled signaling events and that ligand binding to only selected epitopes of MHC class II molecules can induce IL-6 secretion by brain endothelial cells. Differences in the affinity of enterotoxins for MHC class II may also explain the higher response observed with SEA (31). Our results indicate that MHC class II engagement in IFN-γ-pretreated rat brain microvesSEL endothelial cells can also induce cytokine secretion, as previously reported in hemopoietic cells. Together with the previous observation of IL-6 and IL-8 production by human rheumatoid synoviocytes in response to SEA (12), this study demonstrates that MHC class II molecules ectopically expressed in nonhemopoietic cells can trigger cytokine secretion.

Two distinct signaling pathways have been shown to be activated by MHC class II engagement in professional APCs: increase in cAMP accumulation together with activation of PKC in resting B cells (25) and protein tyrosine kinase activation followed by phosphoinositide hydrolysis and calcium mobilization in mono-
Acknowledgments

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References


