INFECTION AND IMMUNITY, June 1997, p. 2000–2005 Vol. 65, No. 6
0019-9567/97/$04.00+0

Functional Analysis of *Mycoplasma arthritidis*-Derived Mitogen Interactions with Class II Molecules

CHANTALE BERNATCHEZ,[1] REEM AL-DACCAK,[1] PIERRE ETONGUÉ MAYER,[1] KHALIL MEHINDATE,[1] LOTHAR RINK,[2] SALAH MECHERI,[3] AND WALID MOURAD[1]*

Centre de Recherche en Rhumatologie Immunologie, Centre Hospitalier de l'Université Laval, Sainte-Foy, Québec, Canada G1V 4G2[1]; Institute of Immunology and Transfusion Medicine, University of Lübeck Medical School, Lübeck, Germany[2]; and Unité d'Immuno-allergie, Institut Pasteur, Paris, France[3]

Received 23 July 1996/Returned for modification 30 September 1996/Accepted 13 March 1997

The ability of superantigens (SAGs) to trigger various cellular events via major histocompatibility complex (MHC) class II molecules is largely mediated by their mode of interaction. Having two MHC class II binding sites, staphylococcal enterotoxin A (SEA) is able to dimerize MHC class II molecules on the cell surface and consequently induces cytokine gene expression in human monocytes. In contrast, cross-linking with specific monoclonal antibodies or T-cell receptor is required for staphylococcal enterotoxin B (SEB) and toxic shock syndrome toxin 1 (TSST-1) to induce similar responses. In the present study, we report how *Mycoplasma arthritidis*-derived mitogen (MAM) may interact with MHC class II molecules to induce cytokine gene expression in human monocytes. The data presented indicate that MAM-induced cytokine gene expression in human monocytes is Zn^{2+} dependent. The MAM-induced response is completely abolished by pretreatment with SEA mutants that have lost their capacity to bind either the MHC class II α or β chain, with wild-type SEB, or with wild-type TSST-1, suggesting that MAM induces cytokine gene expression most probably by inducing dimerization of class II molecules. In addition, it seems that SEA and MAM interact with the same or overlapping binding sites on the MHC class II β chain and, on the other hand, that they bind to the α chain most probably through the regions that are involved in SEB and TSST-1 binding.

Staphylococcal enterotoxins A to E (SEA to SEE), toxic shock syndrome toxin 1 (TSST-1), and *Mycoplasma arthritidis*-derived mitogen (MAM) are members of the bacterial superantigens (SAGs). They have drastic modulatory effects on the immune system which result from their structural-functional interactions with their natural receptors, the major histocompatibility (MHC) class II molecules (18, 36), and the subsequent interaction with T-cell receptors that bear specific Vβ elements (35). Crystallographic, biochemical, and molecular studies revealed considerable information about the sites as well as the mode of interaction of staphylococcal SAGs with class II molecules (7, 8, 16, 25, 27, 38, 42). The staphylococcal SAG TSST-1 contact surface with the HLA-DR α chain is similar to that of SEB, but with an additional extension over the peptide binding groove which partially interacts with bound peptides and contacts the HLA-DR β chain (23, 26). SEA bears two distinct binding sites; the first is located in the C terminus and interacts with high affinity with the HLA-DR β chain, whereas the second is located in the N terminus and is bound to the HLA-DR α chain with low affinity (1, 22, 34). Binding of SEA to the β chain is coordinated by Zn^{2+} ion, whereas TSST-1, SEB, and SEA binding to the α chain is Zn^{2+} independent. The two SEA class II binding sites cooperate with one another; the interaction of the low-affinity site seems to be stabilized by the interaction of the high-affinity one (22). Having two class II binding sites, SEA is able to dimerize MHC class II molecules on the cell surface of monocytes and consequently induces cytokine gene expression (29). In contrast, cross-linking with specific antibodies or T-cell receptor is required for SEB and TSST-1 to induce a similar response (30). Indeed, the simultaneous ligation of class II molecules on the cell surface was reported recently as essential for effective SEA activity (39).

The recently resolved nucleotide and amino acid sequences of MAM revealed that this SAG does not seem phylogenetically or structurally related to other SAGs (10). However, it bears short regions of sequence homology that may predict functional domains that are common to several SAGs, notably, the MAM 11 to 38 region that aligns two regions of SEB that are involved in its interactions with MHC class II molecules (23, 24). MAM also bears a region of similarity with the conserved retroviral sequence of mouse mammary tumor virus 7 (6). This retroviral region is implicated in mouse mammary tumor virus 7 binding to MHC class II molecules, and a similar peptide can competitively block the binding of SEA (40). Although it is well established that MAM binds the class II α chain (5, 13), little is known about the interactions that control its superantigenic activity. A recent study by Driessen and collaborators showed that high levels of Zn^{2+} inhibit MAM, SEA, and SEE activity, suggesting that this ion can regulate MAM reactivity (17). In addition, these results may also suggest a possible implication of class II β chain in MAM interactions with class II molecules.

This study addresses the above possibilities and demonstrates that MAM superantigenic activity via class II molecules is Zn^{2+} dependent, since this ion seems to coordinate its binding to class II molecules. In addition, we suggest that MAM bears two class II binding sites similar to those of Zn^{2+}-coordinated staphylococcal SAGs that permit the dimerization of two class II molecules on monocytes and the consequent induction of cytokine gene expression. The sites of interaction of MAM with both α and β chains of class II molecules seem to

* Corresponding author. Mailing address: CRRI, CHUL, Room 9800, 2705 Blvd. Laurier, Ste-Foy, Québec, Canada G1V 4G2. Phone: (418) 654-2772. Fax: (418) 654-2765.

overlap with those involved in the interactions of staphylococcal SAGs.

MATERIALS AND METHODS

MAM. The MAM used in this study was prepared as previously described (MAM L.R.) (2) and was proven to be lipopolysaccharide (LPS) free with C3H/HeJ (MAM-responder and LPS-nonresponder) and C57BL/6 (MAM-nonresponder and LPS-responder) mice. The MAM preparations are defined as reactive for the SAG and free of LPS when a response is detected in C3H/HeJ culture and absent in C57BL/6 culture. The parameter for responsiveness is proliferation of spleen cell cultures and cytokine induction in murine monocytes. Two additional preparations were obtained as generous gifts from B. Cole (MAM B.C.) and Toxin Technologies (MAM T.T.).

Generation and purification of recombinant wild-type SEA, SEB, and TSST-1. The SEA gene was generated from *Staphylococcus aureus* as previously described (28). The SEB gene (22) was a generous gift from J. D. Fraser (Department of Molecular Medicine, University of Auckland, Auckland, New Zealand), and the TSST-1 gene (15) was obtained from E. Choi (Department of Molecular Genetics, University of Cincinnati, Cincinnati, Ohio). Recombinants were produced in *Escherichia coli* by using the expression vector pGEX-2T for SEA and SEB and the pT7-7 expression vector for TSST-1. SEA and SEB were purified by affinity chromatography on glutathione-agarose (Pharmacia Biotech) as previously described (28). Toxins were released from the fusion protein by cleavage with bovine thrombin (Sigma Diagnostics, Mississauga, Ontario, Canada). The thrombin was removed by a 30-min incubation with a *p*-aminobenzamidine-agarose bead gel (Sigma Diagnostics) and centrifugation. Recombinant TSST-1 was purified from periplasmic contents of *E. coli* cells by a two-step purification procedure (15). The first step consists of using Sep-pak gel to desalt and enrich the TSST-1 product, and in the second step we used a C_{18} reverse-phase high-pressure liquid chromatography column. To remove any residual LPS contamination, SEB and TSST-1 were passed over an endotoxin-removing gel (Pierce, Rockford, Ill.), dialyzed, filtered, and kept frozen at −20°C. No LPS was detected when the toxin preparations were tested by *Limulus* amoebocyte lysate test (sensitivity, 1 ng/mg). The purity of these recombinants was confirmed by high-pressure liquid chromatography and sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by immunoblotting with specific monoclonal antibodies.

Generation of recombinant SEA mutants. Three SEA mutants were generated as previously described (29): (i) mutants in which the aspartic acid at position 227 was substituted by alanine (SEA_{D227A}); (ii) mutants in which the phenylalanine at position 47 was substituted by alanine (SEA_{F47A}); and (iii) double mutants in which F47 and D227 were substituted by alanines ($SEA_{F47A/D227A}$) by using oligonucleotide primers and PCR. A unique restriction enzyme site was introduced into each mutant to enable its identification. Each mutation was confirmed by sequencing the relevant portion of the gene. Recombinants were then produced in *E. coli* by using expression vector pGEX-2T and purified as described above.

Cell line. The THP-1 monocytic human cell line was obtained from the American Type Culture Collection and maintained in RPMI 1640 medium containing 10% fetal calf serum and antibiotics. This cell line expresses low levels of HLA-DR molecules, while it is completely negative for HLA-DQ and HLA-DP (43). To induce high levels of HLA-DR, -DQ, and -DP molecules, the cells were treated with gamma interferon (IFN-γ) (20 U/ml) for 48 h. Prior to use, the cells were washed twice with Hanks balanced salt solution (HBSS). Representative levels of expression of HLA-DR, -DQ, and -DP after such treatment are shown below (see Fig. 2B).

Northern (RNA) blot analysis. Stimulation conditions for each experiment are detailed in the appropriate figure legends. RNA was purified by using Trizol reagent (Gibco BRL Products, Life Technologies, Burlington, Canada) according to the manufacturer's procedure, and 10 μg of RNA was loaded onto 1% agarose gels. The RNA was then transferred onto Hybond-N filter paper and was hybridized with random-primer-labeled cDNA probes for interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α). Equal loadings of RNA were confirmed by hybridization with a glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA probe. The mRNA hybridizing with the cDNA probes was visualized by autoradiography (33).

Fluorescence-activated cell sorter analysis. IFN-γ-treated THP-1 cells were washed twice and resuspended in HBSS or in HBSS containing 10 μM EDTA. After 10 min of incubation at room temperature, the cells were washed and incubated with anti-HLA-DR (L243), anti-HLA-DQ (BT3.4), or anti-HLA-DP (B7.21) for 30 min at 4°C. Unbound antibodies were removed by a washing, and the cells were then incubated with fluorescein isothiocyanate-labeled anti-mouse immunoglobulin. The cells were then washed and analyzed by flow cytometry.

RESULTS

MAM superantigenic activity via class II molecules is Zn^{2+} coordinated. THP-1 is a human monocytic cell line that expresses low levels of HLA-DR and transcribes and synthesizes several cytokines in response to various stimuli, in a manner similar to that of peripheral human monocytes (41). Stimulation of THP-1 cells with staphylococcal SAGs induces, via MHC class II molecules, gene expression of a variety of cytokines (30, 41). However, pretreatment with IFN-γ is absolutely required for MAM-induced cytokine gene expression in these cells, suggesting that maximal class II expression (HLA-DR, -DQ, and -DP) or an IFN-γ priming effect is required for a MAM-induced response (2, 3). Accordingly, all the following experiments were carried out with cells treated for 48 h with IFN-γ (20 U/ml). Since recombinant MAM is not available, we compared the capacity of our MAM preparation (MAM L.R.) to induce IL-1β and TNF-α gene expression in IFN-γ-treated THP-1 cells with those of two other MAM preparations, MAM B.C. and MAM T.T. Figure 1 shows that the three different MAM preparations are able to induce comparable levels of both IL-1β and TNF-α mRNAs. Accordingly, our experiments were carried out with MAM L.R.; the response induced by a 1/100 dilution of this preparation is comparable to that induced by 1 μg of MAM T.T. We evaluated the amount of MAM protein in the MAM L.R. preparation by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, using MAM T.T. as a standard. This preparation was found to contain approximately 70 μg/ml, and therefore its 1/100 dilution, used in this study, contains approximately 0.7 μg/ml.

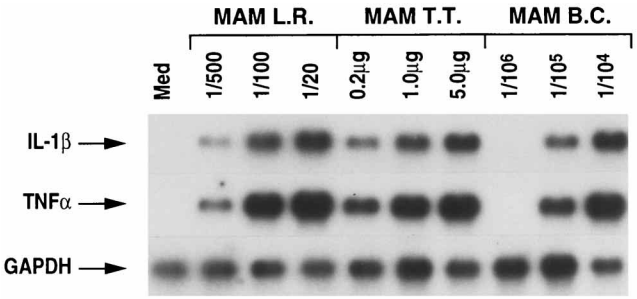

FIG. 1. Induction of IL-1β and TNF-α mRNA expression by three different MAM preparations. IFN-γ-treated cells were washed twice with HBSS, resuspended in RPMI 1640 medium (5×10^6 cells/ml), and stimulated for 1 h at 37°C with different dilutions (as indicated above the lanes) of MAM prepared as previously described (2) (MAM L.R.) or obtained from Toxin Technologies (MAM T.T.) or B. Cole (MAM B.C.). The cells were then harvested, RNA was extracted, and 10 μg of total RNA was loaded in each lane, electrophoresed, transferred to nitrocellulose filters, and hybridized with probes for IL-1β and then TNF-α and the housekeeping GAPDH gene as a control for equal RNA loading. Lane Med, medium alone.

A recent report from Driessen and collaborators has proposed that Zn^{2+} may coordinate the interaction between MAM and class II molecules (17). To investigate the importance of Zn^{2+} in the MAM-induced response, IFN-γ-treated THP-1 cells were resuspended in serum-free medium containing 10 μM EDTA. After 10 min of incubation, the cells were washed, resuspended in serum-free RPMI 1640 medium, and stimulated with MAM, SEA, or LPS. Figure 2A shows that stimulation of THP-1 cells with SEA or MAM did not yield any significant induction of either IL-1β or TNF-α, suggesting that MAM, similarly to SEA, requires the presence of certain ions and most probably Zn^{2+} to interact with its receptor and induce cytokine gene expression. The same treatment failed to affect the LPS-induced response, indicating that our experimental conditions have no negative effect on the biological response of these cells. To ensure that the absence of SEA and MAM responses is not due to alterations in the conformation of class II molecules or the biological response that may be caused by EDTA treatment, IFN-γ-treated THP-1 cells were

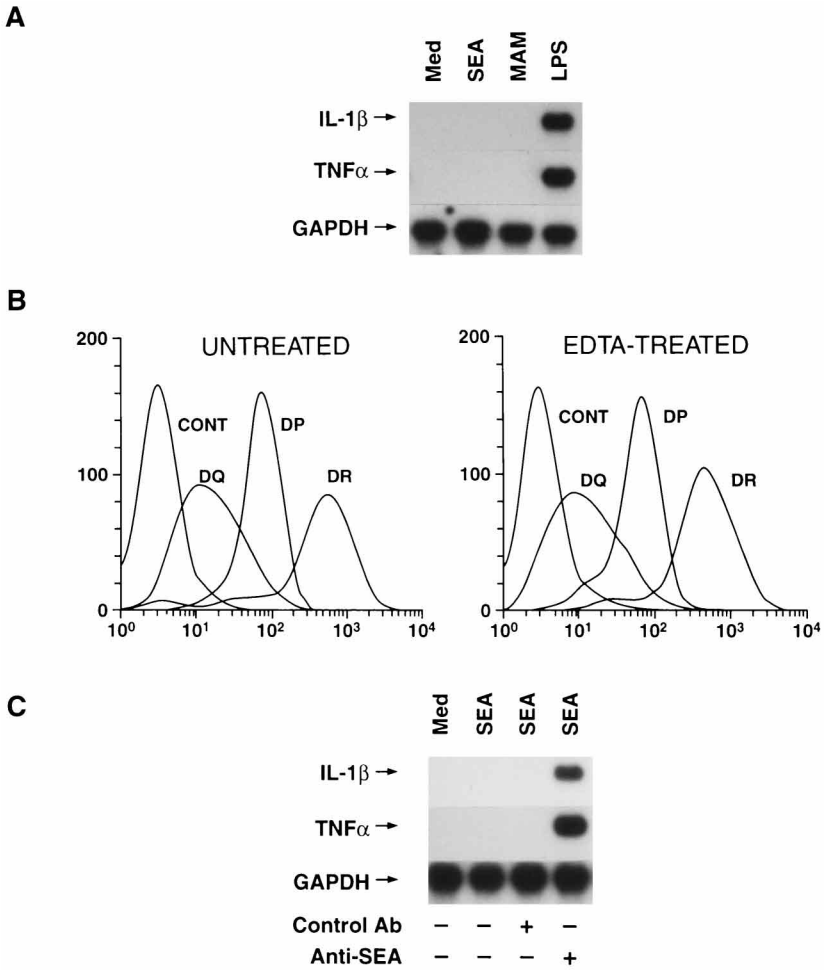

FIG. 2. MAM-induced IL-1β and TNF-α mRNAs are ion coordinated. (A) IFN-γ-treated cells were washed twice with HBSS and resuspended in serum-free medium (5×10^6/ml) containing 10 μM EDTA for 10 min. The cells were then extensively washed with HBSS, resuspended in RPMI 1640 medium, and then stimulated with MAM (1/100), SEA (1 μg/ml), or LPS (1 μg/ml) for 1 h at 37°C. The reaction was stopped, total RNA was purified, and the levels of IL-1β and TNF-α mRNAs were determined as described for Fig. 1. (B) EDTA-treated and -untreated cells were washed, and HLA-DR, -DP, and -DQ expression was analyzed by using specific antibodies. CONT, control. (C) EDTA-treated cells were washed with HBSS, resuspended in RPMI 1640 medium, and stimulated with SEA (1 μg/ml). After 1 h, anti-SEA antibody was added, and the cells were incubated for another 1 h. The reaction was stopped, and the levels of IL-1β and TNF-α were determined as described for Fig. 1. Ab, antibody. Lanes Med, medium alone.

pretreated with 10 μM EDTA for 10 min and washed and then class II expression was verified by using anti-HLA-DR, -DQ, and -DP monoclonal antibodies. Figure 2B shows that all the antibodies used interact in similar fashions with EDTA-treated and -untreated cells, indicating that this treatment does not affect the conformation of class II molecules. EDTA treatment abolishes the capacity of SEA to bind the class II β chain and consequently prohibits the simultaneous ligation of two class II molecules required for cytokine gene expression. However, under these conditions, SEA preserves its capacity to interact with the class II α chain, and cross-linking with an anti-SEA antibody allows dimerization of class II molecules and results in a significant response (Fig. 2C). These results demonstrate that the described EDTA treatment does not affect the signal transduction via class II molecules.

Finally, to confirm the role of Zn^{2+} in the MAM response, IFN-γ-treated THP-1 cells were incubated for 10 min with EDTA (10 μM), extensively washed with HBSS, resuspended in serum-free RPMI 1640 medium alone or supplemented with 1 μM Zn^{2+}, and then stimulated with SEA, MAM, or LPS. Figure 3 shows that addition of 1 μM Zn^{2+} restores MAM- and SEA-induced responses, confirming that both responses are Zn^{2+} dependent. Accordingly, MAM, at least functionally, can be considered among Zn^{2+}-coordinated SAGs.

MAM dimerizes class II molecules by interacting with their α and β chains. Recent functional analysis of the interactions between staphylococcal SAGs and class II molecules provided clear evidence that dimerization of class II molecules on the surface of THP-1 cells is an absolute requirement for staphylococcal-SAG-induced cytokine gene expression (29, 30). As MAM per se is able to induce cytokine gene expression in a Zn^{2+}-dependent manner, it is highly possible that it has the capacity to induce dimerization of class II molecules, most probably through two class II binding sites. To verify this possibility, the effects of SEA mutants that had lost their binding site for either the class II α chain (SEA_{F47A}) or the β chain (SEA_{D227A}) on MAM-induced IL-1β or TNF-α gene expression were determined. Figure 4 shows that preincubation of THP-1 cells with either SEA_{F47A} or SEA_{D227A} prior to their stimulation with MAM completely abolished the MAM-induced gene expression. The inhibitory effect of the mutants used is not due to negative signal, since the same treatment

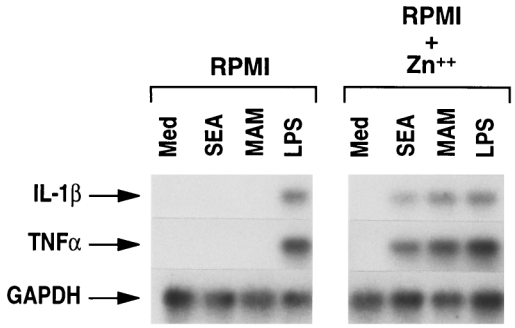

FIG. 3. MAM-induced IL-1β and TNF-α mRNAs are Zn^{2+} coordinated. IFN-γ-treated cells were washed twice with HBSS, incubated with 10 μM EDTA for 10 min, washed, resuspended in serum-free RPMI 1640 medium (5×10^6 cells/ml) alone or supplemented with 1 μM Zn^{2+}, and then stimulated with MAM (1/100), SEA (1 μg/ml), or LPS (1 μg/ml) for 1 h at 37°C. The reaction was stopped, and IL-1β and TNF-α mRNA expression was determined by Northern blotting as described for Fig. 1. Lanes Med, medium alone.

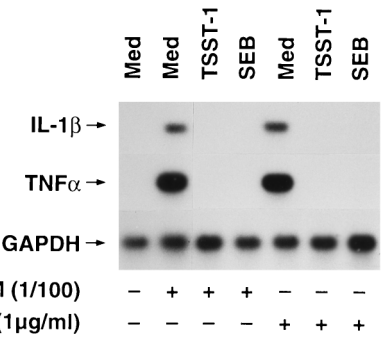

FIG. 5. SEB and TSST-1 inhibit MAM-induced IL-1β and TNF-α gene expression. IFN-γ-treated, HBSS-washed cells were resuspended in RPMI 1640 medium (5×10^6 cells/ml), treated for 30 min with medium alone or SEB or TSST-1 (10 μg/ml), and then stimulated with SEA or MAM for 1 h at 37°C. The reaction was stopped, and IL-1β and TNF-α mRNA expression was determined by Northern blotting as described for Fig. 1. Med, medium.

failed to affect the LPS-induced response (29). Preincubation with SEA mutated at both positions ($SEA_{F47A/D227A}$) did not have any effect on this MAM response. These results suggest strongly that MAM behaves similarly to SEA and bears two binding sites that can interact with two different class II molecules: one is involved in binding to the β chain, while the other interacts with the α chain. Blocking either binding site results in the failure of MAM to induce cytokine gene expression. Our results, obtained by a functional assay, strongly suggest that MAM, similarly to other Zn^{2+}-coordinated SAGs, induces its response by dimerization of class II molecules. In addition, the observed inhibitory effect of SEA mutants suggests that the sites of interaction of MAM with class II molecules overlap with those involved in SEA binding. However, conformational changes or steric hindrance that might be induced upon SEA binding to class II molecules and prohibit MAM binding to these molecules cannot be excluded.

On the basis of the crystal structures of SEB/DR-1 and TSST-1/DR-1 (23, 26) and data on the interaction of SEA with the α chain of the class II molecules (22), it is evident that common residues of the class II α chain mediate the binding of all these toxins. Therefore, to further verify the possible overlap of MAM class II binding sites on the α chain with those of SEB and TSST-1, we evaluated the effect of preincubating THP-1 cells with SEB or TSST-1 prior to their stimulation with MAM. Both toxins were able to block MAM-induced IL-1β and TNF-α gene expression (Fig. 5) in the same manner in which they blocked the SEA-induced response. Accordingly, it is highly likely that MAM interacts, if not with the same sites of class II α chain, with sites that overlap with those involved in the interactions of SEA, SEB, and TSST-1.

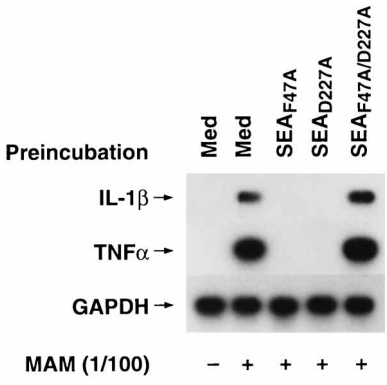

FIG. 4. SEA_{F47A} and SEA_{D227A} inhibit MAM-induced IL-1β and TNF-α gene expression. IFN-γ-treated, HBSS-washed cells were resuspended in RPMI 1640 medium (5×10^6 cells/ml); treated for 30 min with medium alone (lanes Med), SEA_{F47A}, SEA_{D227A}, or $SEA_{F47A/D227A}$ (10 μg/ml); and then stimulated with MAM for 1 h at 37°C. The reaction was stopped, and IL-1β and TNF-α mRNA expression was determined by Northern blotting as described for Fig. 1.

DISCUSSION

Although the superantigenic properties of MAM (2, 4, 9, 14) suggest that this SAG may be an ideal model for studying the role of SAGs in pathogenesis of human autoimmune diseases, especially those characterized by hypergammaglobulinemia, little is known about MAM molecular interactions that lead to T-cell and class II-positive-cell activation. For mice, it is well established that the reactivity to MAM is strictly dependent on the MHC class II haplotype (11, 12). In humans, recent studies with peripheral blood mononuclear cells showed that cytokine induction by MAM was individually restricted (32), suggesting the influence of class II haplotype on the MAM response. On the other hand, it has been shown that MAM induces different Vβ repertoires, depending on the class II HLA-DR haplotype presented, which indicated that the Vβ specificity of MAM is determined by the complex of MAM and class II HLA-DR (31). Collectively, in addition to the importance of MAM in providing a possible link between the genetic backgrounds of autoimmune diseases, such as rheumatoid arthritis, and infectious organisms, these data point out the importance of defining the molecular interactions of this SAG with class II molecules. This study, by its indirect but functional analysis, highlights some aspects of MAM molecular interactions with MHC class II molecules that lead to cytokine gene expression in the human monocytic cell line THP-1.

The influence of Zn^{2+} ion on the superantigenic activity of MAM in peripheral blood mononuclear cells and whole-blood cultures in which both monokine production (IL-1β) and T-cell activation were affected has been reported previously (17). Accordingly, it was suggested that this ion can affect either MAM binding to MHC class II molecules, its recognition by T-cell receptor, or both. Our functional analysis showed that Zn^{2+} ion is indeed required for MAM superantigenic activity that leads to cytokine gene expression via MHC class II molecules in monocytes. These data permit the conclusion that Zn^{2+} regulates MAM binding to MHC class II molecules and

demonstrate that the MHC class II binding site of MAM is most probably structurally similar to the MHC binding region of SEA where Zn^{2+} ion interacts and mediates the binding of both SAGs. The latter possibility is strongly supported by the results obtained with SEA mutants. The inhibition of the MAM-induced response by SEA_{F47A}, which binds only to the β chain, supports the implication of class II β chain in the binding of MAM. On the basis of amino acid sequence, the β chains of MHC class II (HLA-DR, -DQ, and -DP) bear Zn^{2+} binding motifs that can be involved in the binding of Zn^{2+}-coordinated SAGs (19). Accordingly, the MAM Zn^{2+}-coordinated binding to class II molecules points out, for the first time, that this SAG has the ability to bind the β chain of these molecules in addition to its well-established capacity to bind the α chain (5, 13) and indicates that MAM bears two distinct MHC class II binding sites.

Results obtained in recent years have given ample evidence that several cell surface receptors, including growth factors and cytokine receptors, are activated by ligand-induced dimerization or oligomerization and that this mechanism is of general applicability for the regulation of signal transduction (20). Cytokine gene expression induced by staphylococcal SAGs upon their ligation of class II molecules is mediated by homodimerization of their receptor (29, 30). Indeed, a recent study by Tiedemann and Fraser demonstrated that ligation of two MHC class II molecules on antigen-presenting cells by a single SEA is essential for effective SAG function (39). The capacity of SAGs to induce dimerization of their receptors is governed, at least in part, by the SAG class II binding sites that orient their interaction with the α and β chains of class II molecules. Our data showed that MAM superantigenic activity is most probably mediated by dimerization of class II molecules on monocyte cell surfaces through two distinct class II binding sites. Although our functional analysis does not permit a precise definition of class II MAM binding sites, the inhibitory effect of SEA mutants as well as that of SEB and TSST-1 on the MAM-induced response permits the prediction of certain possible sites. MAM bears a short region of 27 amino acids (residues 11 to 38) that has some homology with the SEB class II binding site and possibly contains, between residues 12 and 29, certain homology with the SEA class II binding site. Since preincubation of THP-1 cells with SEA_{D227A}, which binds only to the class II α chain, or with SEB completely abolishes MAM-induced IL-1β and TNF-α gene expression, and in view of the homology described above, it is highly likely that the MAM region from amino acids 11 to 38 is implicated in its interactions with class II molecules, in particular the α chain. It is also strongly possible that the class II α-chain MAM-binding residues are the same as or overlap those that bind SEA, SEB, and TSST-1. Several motifs in the MAM primary sequence (10) can form a Zn^{2+} binding motif; however, it is quite difficult to predict those that are implicated in its binding to the class II β chain. In contrast, at least one of the class II β-chain MAM binding regions is most probably that involved in SEA binding, notably the region containing the H81 residue, since this region of class II HLA-DR is Zn^{2+} coordinated (21). Further studies should be done to determine the implication of class II HLA-DQ and HLA-DP β chains. It is worth noting that a MAM Zn^{2+} motif(s) can permit the formation of MAM-MAM homodimers, similarly to SED (37), which can generate multimeric interactions between this SAG and class II molecules. However, no clear evidence concerning this issue is yet available, and further studies should clarify this possibility.

Whether the described mode of interaction between MAM and class II molecules is influenced by the class II haplotype is still to be determined. However, this study, by functional analysis, provides the first description of molecular interactions of MAM with class II molecules. Such a mode of interaction, either solo or in combination with class II haplotype, may have an impact on MAM superantigenic reactivity.

ACKNOWLEDGMENTS

We thank J. D. Fraser for providing the SEB plasmid, E. Choi for providing TSST-1, and B. Cole for providing a MAM preparation.

This work was supported by grants to Walid Mourad from the MRC. W. Mourad and R. Al-Daccak are recipients of scholarships from the FRSQ.

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Editor: J. R. McGhee